Prevention of Human Natural Killer Cell - Mediated Immune Responses in Xenotransplantation

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1. Summary

Xenotransplantation - the transplantation of cells, organs or tissues between members of different species – might have the potential to overcome the severe shortage of human organs available for transplantation. For several reasons the miniature pig is viewed as a suitable source of animal organs for transplantation into human patients. However, the problems associated with xenotransplantation are complex, and include immunological and physiological obstacles. Pre-formed xenoreactive natural antibodies (NAb) in human serum, and an immediate and vigorous rejection reaction, the so-called hyperacute rejection (HAR), can lead to a loss of the porcine organ within hours. Most of these Nab are directed against the galactose-α-1,3-galactose (αGAL), a saccharide structure expressed on the cell surface of all mammals except humans, apes, and Old World monkeys. With the generation of pigs lacking αGAL, HAR has largely been overcome, as evidenced by the transplantation of hearts and kidneys derived from the modified pigs into baboons. A second rejection mechanism, the acute vascular rejection (AVR), is characterized by the infiltration of natural killer (NK) cells, monocytes/macrophages, and neutrophils into the transplant. The role of NK cells in the rejection of xenografts has been demonstrated in various studies, both *in vitro* and *in vivo*. NK cells are large granular lymphocytes with a cytotoxic potential that is tightly regulated by both inhibitory as well as activating receptors that NK cells express on their surface. Inhibitory receptors, such as the members of the CD94 and NKG2 family, recognize major histocompatibility complex (MHC) class I molecules, in this case human leukocyte antigen E (HLA-E), on the surface of potential target cells and therefore deliver a negative signal preventing an NK cell from killing the target cell. On the other hand, activating NK cell receptors, such as natural cytotoxicity receptors (NCR) and NKG2D, promote the ability of NK cells to kill a potential target cell after having encountered their corresponding, as yet unidentified, ligands on the surface of a target cell. Consequently, NK cytotoxicity occurs if stimulatory signals outweigh inhibitory signals derived from a potential target cell. Porcine endothelial cells (pEC) are susceptible to human NK cell-mediated lysis, possibly due to the inability of porcine MHC class I molecules to signal through human NK cell inhibitory receptors.

Furthermore, pEC are also known to express ligands for activating human NK cell receptors.

The aims of this study were: (1) to identify those activating receptors on human NK cells that are involved in xenogeneic NK cell-mediated cytotoxicity against pEC; (2) to identify the porcine ligand(s) for two activating receptors of the human NK cells, namely NKG2D and NKp44; and (3) to study whether transgenic expression of HLA-E can protect pEC from human NK cell-mediated xenogeneic cytotoxicity.

It is demonstrated in this study that human NK cells mediate xenogeneic cytotoxicity against porcine cells through activating receptors NKG2D and NKp44, but not through NKp30 and NKp46. Furthermore, cytotoxicity of human NK cell clones against the immortalized pEC line PEDSV.15 strongly correlates with the NKp44 expression levels. Xenogeneic antibody-dependent cell-mediated cytotoxicity using freshly isolated NK cells and autologous human serum is not prevented by blocking NKG2D. It was further determined that the homologue of human UL16-binding proteins (ULBP), pULBP1, but not the homologue of the human MHC class I chain-related (MIC) protein, pMIC2, serves as the predominant, if not the only, functional ligand for the human NKG2D on porcine cells. It was also shown that the level of protection of pEC against human NK cell-mediated cytotoxicity depends both on the expression levels of HLA-E and on the respective NK cell receptor CD94/NKG2A. Furthermore, the observed effect was specifically mediated by HLA-E expression and can be reversed by blocking of its receptor CD94/NKG2A. In addition, pEC derived from HLA-E/β2-microglobulin-transgenic pigs are efficiently protected against human NK cell-mediated cytotoxicity.

These studies suggest that elimination of pULBP1 and an as yet unidentified NKp44 ligand on porcine tissues represent attractive possibilities to protect porcine xenografts from human NK cell responses, including direct xenogeneic cytotoxicity. In addition, transgenic expression of HLA-E on the surface of porcine cells might contribute to the successful prevention of NK cell-mediated damage in clinical xenotransplantation. Future studies should focus on other detrimental ways, with which NK cells target pEC, such as IFN-γ release, activation of pEC, and recruitment of other cells of the immune system.

2. Zusammenfassung

Xenotransplantation – die Transplantation von Zellen, Organen oder Geweben über die Speziesgrenze hinweg – könnte das Potential besitzen den gravierenden Mangel an Organen für die Transplantation zu überwinden. Aus verschiedenen Gründen wird das Miniaturschwein als eine passende Quelle für tierische Organe für die Transplantation in humane Patienten gesehen. Trotzdem sind die Probleme, welche mit der Xenotransplantation assoziiert sind komplex und beinhalten sowohl immunologische als auch physiologische Hürden. Präformierte xenoreaktive natürliche Antikörper (NAb) im humanen Serum und eine sofortige und heftige Abstossungsreaktion, die so genannte hyperakute Abstossung (HAR), kann zu einem Verlust des porzinen Organs innerhalb von Stunden führen. Die meisten dieser NAb sind gegen die Galaktose-α-1,3-Galaktose (αGAL) gerichtet, eine Zuckerstruktur auf der Zelloberfläche aller Säugetiere, mit der Ausnahme von Menschen, Menschenaffen und Meerkatzen. Durch die Herstellung von Schweinen denen αGAL fehlt, konnte die HAR mehrheitlich überwunden werden. Dies wurde mittels der Transplantation von Herzen und Nieren solcher modifizierter Schweine in Paviane gezeigt. Ein zweiter Abstossungsmechanismus, die akute vaskuläre Abstossung (AVR), ist charakterisiert durch die Infiltration von natürlichen Killerzellen (NK Zellen), Monozyten/Makrophagen und Neutrophilen ins Transplantat. Die Rolle der NK Zellen in der Abstossung von Xenotransplantaten wurde in verschiedenen Studien, sowohl *in vitro* als auch *in vivo*, gezeigt. NK Zellen sind grosse granuläre Lymphozyten mit einem zytotoxischen Potential, das straff reguliert wird durch inhibierende und aktivierende Rezeptoren, welche auf der Oberfläche der NK Zellen exprimiert werden. Inhibitorische Rezeptoren, wie zum Beispiel die Mitglieder der CD94 und NKG2 Familie, erkennen MHC Klasse I Moleküle, in diesem Fall HLA-E auf der Oberfläche potentieller Zielzellen, und senden ein negatives Signal, welches die NK Zelle daran hindert diese Zielzelle zu töten. Andererseits fördern aktivierende Rezeptoren, wie zum Beispiel natürliche Zytotoxizitäts-Rezeptoren (NCR) und NKG2D, die Fähigkeit von NK Zellen potentielle Zielzellen zu töten nachdem sie den passenden Liganden (in diesem Fall noch unbekannt) auf der Zelloberfläche der Zielzelle gebunden haben. Folglich tritt NK Zell Zytotoxizität dann auf, wenn die stimulierenden Signale die inhibierenden Signale einer potenziellen Zielzelle

überwiegen. Porzine Endothelzellen (pEC) sind wahrscheinlich empfindlich gegenüber humaner NK Zell-vermittelter Lyse, weil die porzinen MHC Klasse I Moleküle nicht über die humanen inhibierenden NK Zell Rezeptoren senden können. Des Weiteren exprimieren pEC Liganden für die aktivierenden humanen NK Zell Rezeptoren.

Die Ziele dieser Studie waren: (1) die Identifizierung der aktivierenden Rezeptoren auf den humanen NK Zellen, welche in der xenogenen NK Zell-vermittelten Zytotoxizität gegen pEC involviert sind; (2) die Identifizierung der porzinen Liganden zweier aktivierender Rezeptoren auf humanen NK Zellen, NKG2D und NKp44; und (3) zu untersuchen, ob die transgene Expression von HLA-E pEC vor der humanen NK Zell-vermittelten xenogenen Zytotoxizität schützt.

Diese Studie hat gezeigt, dass humane NK Zellen die xenogene Zytotoxizität gegen porzine Zellen durch die aktivierenden Rezeptoren NKG2D und NKp44, jedoch nicht durch NKp30 und NKp46, vermitteln. Des Weiteren hat die Zytotoxizität humaner NK Zell Klone gegen die immortalisierte pEC Linie PEDSV.15 stark mit dem NKp44 Expressionslevel korreliert. Xenogene Antikörper-abhängige Zell-vermittelte Zytotoxizität beim Gebrauch von frisch isolierten NK Zellen in Kombination mit autologem humanem Serum wurde nicht verhindert durch die Blockade von NKG2D. Ferner wurde ermittelt, dass das Homolog des humanen UL-16-bindenden Proteins (ULBP), pULBP1, jedoch nicht das Homolog des humanen MIC Proteins, pMIC2, als vorherrschender, wenn nicht einziger, funktioneller Ligand für humanes NKG2D auf porzinen Zellen fungiert. Es wurde auch gezeigt, dass die Höhe des Schutzes der pEC gegenüber humaner NK Zell-vermittelter Zytotoxizität sowohl abhängig von der Expressionshöhe von HLA-E als auch des dazugehörenden NK Zell Rezeptor CD94/NKG2A ist. Des Weiteren war der beobachtete Effekt spezifisch vermittelt durch die HLA-E Expression und konnte aufgehoben werden durch das Blockieren des Rezeptors CD94/NKG2A. Ausserdem waren pEC, die von HLA-E/β2-mikroglobulin-transgenen Schweinen isoliert wurden effektiv geschützt gegenüber humaner NK Zell-vermittelter Zytotoxizität.

Diese Studien weisen darauf hin, dass die Elimination von pULBP1 und bis jetzt unidentifizierter NKp44 Liganden auf porzinem Gewebe eine attraktive Möglichkeit

zum Schutz des porzinen Xenotransplantates vor der humanen NK Zell Antwort, inklusive der direkten Zytotoxizität, darstellt. Ausserdem könnte auch die transgene Expression von HLA-E auf der Oberfläche porziner Zellen zur erfolgreichen Verhinderung der NK Zell-vermittelten Schädigung in der klinischen Xenotransplantation beitragen. Zukünftige Studien sollten sich auf weitere schädliche Wege konzentrieren mittels derer NK Zellen pEC angreifen, wie zum Beispiel die Sekretion von IFN-γ, die Aktivierung der pEC und die Rekrutierung anderer Zellen des Immunsystems.

3. Introduction

3.1. Xenotransplantation

The transplantation of cells, tissues, and organs from animals into humans, so-called xenotransplantation, has been sought of for over 100 years. In fact, the first transplantation experiments in humans used animal organs (1). Interestingly, the reason behind this was that the transplant surgeons did not think human organs could be obtained in sufficient numbers. For similar reasons, today xenotransplantation is considered to be a possible solution for the lack of sufficient human organs for clinical transplantation. In the 16th century, the pioneers of xenotransplantation performed xenotransfusions, *i.e.* the transfusion of sheep blood into patients. First cell and tissue xenotransplantations were reported in the early 20th century. During the 1960s, kidneys, hearts, and livers of primates were transplanted by different groups, most notably by Reemtsma (2) and Starzl (3), with moderate success. One of the most successful clinical xenotransplantations took place in 1964 and was performed by the group of Reemtsma. In one patient a chimpanzee kidney survived with adequate function for nine months (2). These early clinical efforts on xenotransplantation were driven both by the mortality of end-stage organ failure unless organ transplantation was performed, and by a relative lack of human organs for that purpose. In the later part of the 1960s, these factors changed with the greater availability of hemodialysis and the acceptance of the concept of brain death. Thus, interest in xenogeneic transplantation diminished over the next 15 years, while allogeneic transplantation of heart, lung, liver, kidney, and pancreas became fully established. Great progress has also been achieved in terms of surgical techniques, immunosuppressive regimens, and pre-transplant diagnostic tools, all of which have contributed to increased patient and graft survival after allotransplantation. Nevertheless, the increasing success of allogeneic transplantation led to renewed interest in xenotransplantation, due to the insufficient supply of human organs for the increasing number of patients that would benefit from transplantation.

For several reasons the miniature pig may be a potential organ source, despite a relatively high phylogenetic distance to humans (4, 5). Pigs breed rapidly and in large numbers, which guarantees an almost unlimited availability of suitable and cost-effective organs. Furthermore, they can be housed in pathogen-free environments,

which limits the risk of infections of the recipient. In addition, their organs share anatomic and physiologic similarities with human organs. Lastly, the public acceptance of the use of pig organs in medicine is expected to be wide, since pigs are also bred for the nutrition industry. In contrast, primates that were considered to be the best potential donors for xenotransplantation because of their close genetic relation to humans, would hardly be accepted as organ source in the public. Furthermore, they are difficult to breed in large numbers and they are of small size. Beside the advantages of xenotransplantation, huge immunological barriers still have to be overcome, since xenotransplantation excites nearly every inflammatory, immune and coagulation pathway (6).

In principle, there are two distinct types of xenotransplants: primary and secondary vascularized transplants. Primary vascularized organs, such as the heart, kidney, liver, or lung, have to get connected directly to the blood vessels of the recipient in order to function properly. Therefore, in these cases, the immediate exposure of donor vascular endothelium to the recipient's circulation determines the character of the initial immune response (7). In contrast, secondary vascularized xenotransplants, such as islets and skin, or cell transplants including bone marrow cells, neural cells, or hepatocytes (8), get vascularized at a later time point and therefore will cause distinct biological reactions. Because donor endothelium is not involved in the latter cases, several of the immunologic responses that arise against primary vascularized xenografts will not occur.

Furthermore, the degree of relationship between donor and recipient can be discriminated. Transplantations between phylogenetically closely related species, for example human and baboon, are concordant. Usually there are no preformed xenoreactive antibodies (Ab) in this species combination and therefore no hyperacute rejection (HAR) will occur. On the other hand, transplantations between species of distant relation, including pig–to-human, are called discordant because the recipient possess circulating preformed donor-reactive Ab (natural Ab; NAb) which induce HAR of the xenograft.

3.1.1. Rejection Mechanisms

The rejection of a xenograft can be divided into different phases, where one or more of these may be involved. Firstly, HAR, a vigorous and irreversible process, may

arise. In the case of pig-to-human xenotransplantation, this process starts within minutes and leads to rejection within hours, caused by binding of preformed xenoreactive NAb to the porcine endothelium followed by complement activation and coagulation (9). If HAR is prevented, acute vascular rejection (AVR) may be induced within hours and lead to rejection within days. This step is represented by a sequence of events, including activation and recruitment of several leukocyte populations, and activation of the graft endothelium (10). The next hurdle, which may be induced after several days, is mediated by T cells. Despite previous reports that cellular immunity might be less of a barrier to xenografts than it is to allografts, in the most relevant species combination for potential clinical xenografting (*i.e.* pig to primate) the cellular responses *in vitro* are as great or greater than the corresponding allogeneic responses (11).

3.1.1.1. Hyperacute Rejection

A primary vascularized, discordant xenotransplant will immediately be rejected after transplantation (Figure 1). The histology of this type of rejection is marked by extensive intravascular thrombosis and extravascular hemorrhage, which is similar to the rejection in ABO-incompatible allografts. This observation led to the idea that it was primarily an Ab-mediated lesion (12). The binding of Ab to the graft leads to complement activation, which is a critical mediator of graft injury (13). Therefore, complement regulatory proteins, including DAF (CD55), MCP (CD46), and CD59, also play a critical role in determining the intensity of HAR. However, these proteins often do not regulate the function of complement proteins from other species (14).

An important finding was that pig endothelium expresses a single dominant epitope, the galactose-α-1,3-galactose (αGAL), which is responsible for the binding of a large portion (approximately 85%) of the human NAb (15). This determinant is formed by the action of the α-1,3-galactosyltransferase (α1,3GT), which glycosylates N-acetyllactosamine. On the other hand, humans and all higher order primates after the New World monkeys do not have a functional gene for this transferase and instead use α-1,2-fucosyltransferase to form the H substance from the substrate. It is believed that these species produce anti-αGAL Nab as a result of the colonization of the gut by bacteria and of the exposure to viruses, protozoa, or components in food carrying αGAL antigens (16). Although the αGAL determinant appears to bind the

majority of preformed human NAb, it is not the only pig determinant that binds human Nab (17).

Binding of xenoreactive NAb to porcine endothelial cells (pEC) leads to so-called type I endothelial cell (EC) activation, *i.e.* non-transcriptional activation (18). This is a very rapid step and leads to EC retraction, expression of P-selectin and von Willebrand factor, secretion of platelet activating factor and loss of heparan sulfate (19). The observed EC retraction might be caused by rapid redistribution of platelet/EC adhesion molecule (PECAM-1/CD31) and VE-cadherin away from cell junctions as a result of αGAL cross-linking.

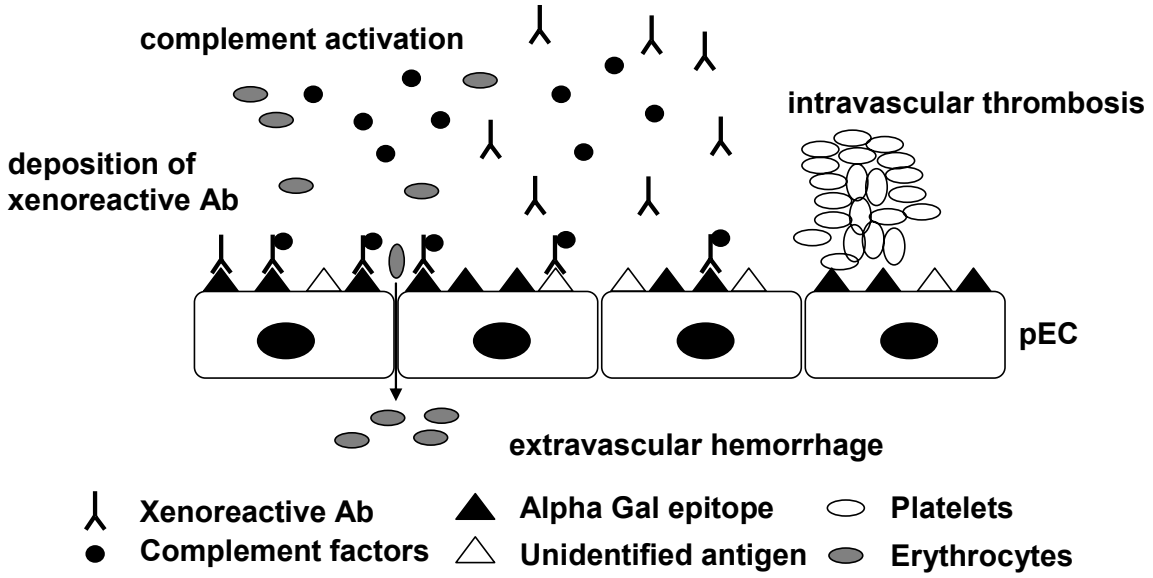

Figure 1: Hyperacute rejection of a porcine xenograft. The binding of Ab (mainly directed against αGAL) to the graft leads to complement activation, which is a critical mediator of the lesion of the graft. The histology of this type of rejection is marked by extensive intravascular thrombosis and extravascular hemorrhage.

3.1.1.2. Acute Vascular Rejection

Leventhal et al. were the first to describe AVR in a guinea pig-to-rat transplantation model (20). The histology of this type of rejection is characterized by EC swelling, ischemia, a diffuse microvascular thrombosis with fibrin deposition, and cellular infiltrates consisting of Natural killer (NK) cells, monocytes/macrophages, and neutrophils (18, 20-23) (Figure 2). A key factor in the pathogenesis of AVR is the so-called type II EC activation (24), *i.e.* transcriptional activation mediated by NF-κB (25). This is characterized by increased transcription of genes encoding prothrombotic molecules such as tissue factor (26), major histocompatibility complex (MHC) class I and II molecules (27, 28), cell adhesion molecules (E-selectin/CD62E,

vascular cell adhesion molecule-I/VCAM-1/CD106, intercellular adhesion molecule-1/ICAM-1/CD54) (29), cytokines and chemokines (interleukin (IL) -1, IL-6, IL-8, monocyte chemoattractant protein/MCP-1) (30), as well as costimulatory molecules of the B7 family (CD80/CD86) (31, 32). On the other hand, thrombomodulin expression is decreased (33). Altogether, these EC changes promote leukocyte recruitment, platelet aggregation, and loss of thromboregulation. Thus, EC are not merely targets of the immune attack, but are central to the pathogenesis of the rejection process by interacting with Ab and regulating leukocyte-EC cross-talk.

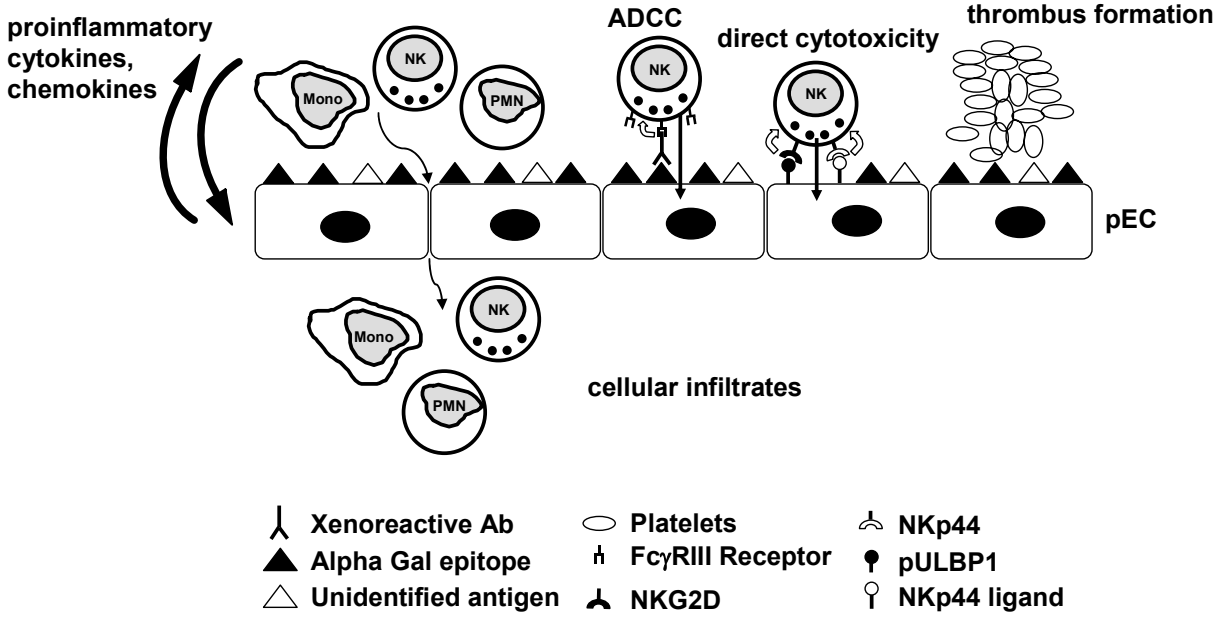

Figure 2: Acute vascular rejection of a porcine xenograft. A key factor in the pathogenesis of AVR is the so-called type II EC activation. The histology of this type of rejection is characterized by EC swelling, ischemia, a diffuse microvascular thrombosis with fibrin deposition, and cellular infiltrates consisting of NK cells, monocytes/macrophages (Mono), and neutrophils (PMN).

3.1.1.3. Role of Cellular Infiltration during Acute Vascular Rejection

Since cellular infiltrates are observed in xenografts during AVR, an important role for the cellular innate immune system is suggested. Some innate cells, such as NK cells (34, 35), monocytes (25, 36), and neutrophils (22), directly recognize and activate pEC. Leukocyte extravasation has been shown to be governed by adhesion molecules belonging to the selectin, integrin, and immunoglobulin protein families in close collaboration with chemokines (37, 38). The current model for leukocyte extravasation is common to different leukocyte subpopulations even though they employ different members of the adhesion protein families. It is divided into four

phases: recruitment of leukocytes by chemotaxis, rolling adhesion, firm adhesion, and transendothelial migration (37) (Figure 3).

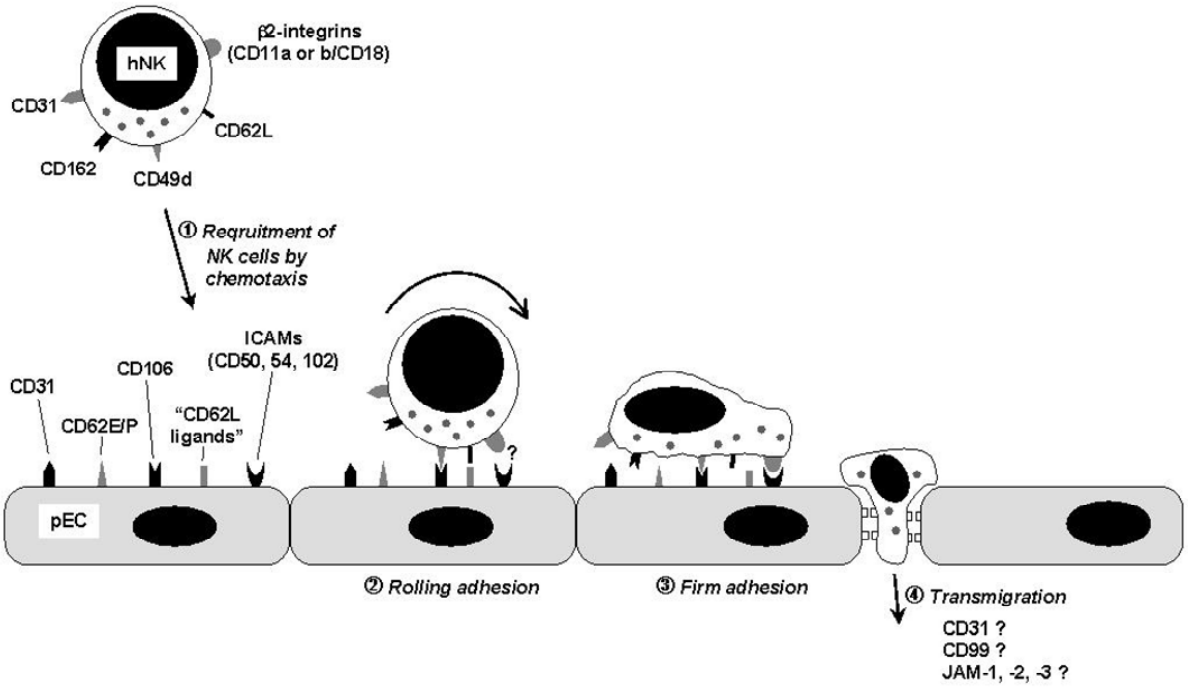

Figure 3: Interaction of human NK cells and pEC (39). Shown is a simplified view of the receptor/ligand interactions involved in chemotaxis, rolling adhesion, firm adhesion, and transmigration.

Following recruitment of leukocytes from the circulation, E- and P-selectin on pEC and L-selectin on leukocytes initiate the rolling phase by binding to carbohydrate counter-receptors on leukocytes and pEC, respectively. Rolling is required for the subsequent firm adhesion, a phase which is carried out by leukocyte integrins (β_1-integrins such as CD49d/CD29, and β_2-integrins such as CD11a/CD18, CD11b/CD18, and CD11c/CD18) binding to pEC immunoglobulin (Ig) superfamily members (VCAM-1, ICAM-1,-2, and -3) (37). The actual transendothelial migration step involves another Ig superfamily member, CD31 (PECAM-1), which facilitates migration through the pEC layer and basal lamina by its homophilic and heterophilic interactions (40, 41).

The most significant phenotypic difference between porcine and human EC is the expression of αGAL. Whereas this molecule is important in the phase of hyperacute graft rejection, its importance in the adhesion step during the AVR remains controversial. An αGAL-dependent adhesion of human NK cells to pEC was suggested by Miyagawa et al. (42, 43), whereas others claim the interaction of

human peripheral blood mononuclear cells (PBMC), polymorphonuclear neutrophils (PMN), and NK cells to be independent of αGAL (35, 44).

Most of the adhesion receptor pairs examined so far seem to be compatible between pig and men (29, 39, 45). This has been shown by cloning of porcine E- and P-selectin as well as by blocking studies using Ab against porcine adhesion molecules to successfully block adhesion of human leukocytes.

3.1.1.4. Role of NK Cells during Acute Vascular Rejection

The role of NK cells in the rejection of both concordant and discordant xenografts has been demonstrated in various studies (46, 47). In several species combination, including pig-to-human, evidence from *in vitro* studies suggests NK cells to lyse xenogeneic cells (43, 46-52). Furthermore, they activate porcine endothelium upon direct contact and act as a potent source for pro-inflammatory cytokines such as interferon-γ (48, 53). Human NK cells have further been demonstrated to adhere to and lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive Ab, by Ab-dependent cell-mediated cytotoxicity (ADCC) (54-56). In addition, pig organs perfused with human blood *ex vivo* are predominantly infiltrated by NK cells (57-59), and NK cells are present in histological samples of graft rejection in concordant and discordant rodent and preclinical pig-to-baboon models (60-62). In particular, studies by Waer et al suggest an important role for NK cells in the rejection of hamster-to-nude rat xenografts (61-63). Also a slight additional prolongation of xenograft survival has been achieved in a small number of studies by the additional depletion of NK cells along with other forms of immunosuppression (64). In other small animal studies, however, CD4+ T cell depletion without NK depletion has led to prolonged xenograft survival, and no effect was seen by the additional depletion of NK cells (65, 66). A possible explanation of this fact is that T-cell derived IL-2 may be necessary to activate NK cells in order to participate in xenograft rejection. There is good evidence that NK cells play an especially important role in resistance to xenogeneic bone marrow engraftment, since allogeneic engraftment can be achieved with T cell-depleting nonmyeloablative conditioning, whereas xenogeneic bone marrow engraftment requires additional anti-NK cell therapy (67).

Birmele et al. reported that about 50% of human peripheral blood lymphocytes adhering to resting pEC were NK cells (68, 69). In this process, a dominant role for

the interactions between CD49d, presumably in combination with CD29, and CD106 is indicated by several studies (39, 70). Furthermore, masking of human CD62L, CD11a/CD18, and CD11b/CD18 by specific Ab also revealed some importance for these molecules. In conclusion, xenogeneic NK cell adhesion depends primarily on CD49d-CD106 interactions in both rolling and firm adhesion.

Under normal physiologic conditions, NK cytotoxicity is accurately regulated to lyse only transformed or infected cells (71). However, pEC are lysed by human NK cells due to the inability of porcine MHC class I molecules, so-called swine leukocyte antigen (SLA) class I, to signal through human NK cell inhibitory receptors, (72). In addition, functional interactions between NKG2D and NKp44 on human NK cells and their corresponding, yet unidentified, ligands on pEC also deliver cytotoxic signals (73). The other two activating receptors on human NK cells (NKp30 and NKp46), however, do not have any corresponding ligands on pEC.

Theoretically, xenogeneic NK cytotoxicity could be avoided either by expressing human leukocyte antigen (HLA) class I molecules on porcine cells or by blocking the function of activating receptors. Indeed, several studies demonstrated that the expression of human MHC class I molecules including HLA-B27, -Cw3, -E, and -G on porcine cells provided partial protection from lysis mediated by polyclonal human NK cells (74-79) (see also results section 5.4. and 5.5). However, complete inhibition of NK cytotoxicity has not been achieved by transgenic HLA class I expression since the corresponding NK inhibitory receptors for each HLA molecule were expressed only on NK subpopulations. Moreover, protection from ADCC by HLA class I expression has been demonstrated only for NK clones, but not for polyclonal NK cells (76, 80). On the other hand, pEC were almost completely protected against human NK cell lysis by the combined blocking of NKG2D and NKp44 receptors by specific monoclonal Ab (mAb) (81) (see also results section 5.1.).

3.1.2. Physiologic Function of Xenogeneic Organs

It is very difficult to estimate, if pig organs are able to function adequately in humans, since to date, only few xenotransplants have survived for prolonged periods. However, it is already known that chimpanzee kidneys are able to support human life and that porcine insulin can regulate blood sugar levels in humans. On the other hand, there are reports that primates surviving with pig kidney transplants develop marked anemia, raising the possibility that pig erythropoietin may not function

properly in primates. And in some species combinations, xenogeneic stem cell engraftment is diminished by the lack of appropriate stem cell growth factors (82-84). These examples indicate that some physiologic functions of xenogeneic organs will remain intact, whereas others might not. On one hand, it could be expected that appropriately sized pig hearts might function in humans, on the other hand, metabolically more complex organs such as the liver may work insufficiently.

3.1.3. Infectious Risks in Xenotransplantation

Initial microbiological concern focused on pig-derived bacteria or parasites. However, the ability to exclude extracellular organisms and bacteria from herds of swine destined for the use as organ donors has focused attention on potential viral pathogens. To date, a number of such pathogens have been identified that may cause a risk of infection by the transmission from the xenograft to the donor. These pathogens include porcine endogenous retrovirus (PERV), porcine cytomegalovirus, and porcine lymphotropic herpesvirus (85). Transplantation poses a unique epidemiologic hazard due to the efficiency of the transmission of pathogens with the graft, facilitated also by the use of immunosuppressive drugs.

Concern about retroviral transmission in xenotransplantation relates to the potential for “silent” transmission, *i.e.* an unapparent infection which may cause altered gene regulation, oncogenesis, or recombination. This may result in an activation of latent virus and the development of clinical manifestations, if any, more than a decade later. So far, only three closely related C-type PERV (PERV A, B, C) have been identified in swine that possess infectious potential (86-89). Two of these, PERV-A and -B, can infect human and pig cells *in vitro*, whereas the third group, PERV-C, infects porcine cells only (90). PERV mRNAs are expressed in all pig tissues and in all breeds of swine tested so far, but size and amount of it differs, consistent with the *in vivo* recombination and/or processing (91). On the other hand, no evidence of infection of human cells has been demonstrated *in vivo* and no disease resulting from this family of viruses has been described in swine or humans to date (92-95). Furthermore, PERV appears to be susceptible to currently available antiviral agents (96).

Activation of latent herpes virus infection during periods of intensive immune suppression or immune dysfunction and by immune reactivity to grafts is an important problem in human allotransplantation (97). Comparable viruses also exist in swine, but tend to be species-specific and therefore would be expected to cause

infection only in host species-derived tissues. A variety of other potential human pathogens have been described in swine, including porcine circovirus, porcine encephalomyocarditis virus, swine influenza viruses, and others. However, none has yet been associated with human diseases.

3.1.4. Therapeutical Strategies to Overcome Xenograft Rejection

Cyclosporine and other immunosuppressive drugs that efficiently inhibit allograft rejection, are not able to control the massive induction of immune responses in pig-to-human xenotransplantation models (98). However, there are strategies shown to be efficient in preventing HAR. These include the removal of xenoreactive NAb by plasmapheresis or immunoabsorption using columns carrying solid phase αGAL oligosaccharides (17), the administration of soluble synthetic α–GAL molecules to block anti-αGAL NAb, and the depletion/inactivation of complement using soluble complement receptor I (99), cobra venom factor (only for rodents and baboons), and mAb directed against complement proteins (100). However, all these therapies influence the graft recipient. One great advantage of xenotransplantation is that xenogeneic organs may be adapted before transplantation. Since techniques for the generation of transgenic and knockout animals have improved a lot in the recent years, there are nearly unlimited possibilities for strategies to prevent xenorejection. For these reasons, huge efforts to identify potential molecules involved in the process of rejection are going on, with the aim of remodelling or eliminating them. On the other hand, also molecules with the potential to protect a xenograft need to be identified in order to transgenically express them on pig organs.

To date, a surface molecule on pEC, αGAL, has been identified as a major factor leading to HAR (15) (see chapter 3.1.2.1). In the year 2003, αGAL-deficient pigs were generated by the elimination of both functional alleles of the α1,3GT gene (101, 102). Hearts and kidneys of these animals showed a significantly prolonged survival in baboon recipients even in the presence of complement and Nab (103-105). These studies showed that HAR may be overcome by the use of organs from αGAL-deficient pigs. Nevertheless, non-α-Gal-specific NAb in human serum may still lead to complications. The identification of these Nab and their ligands is currently in progress. Another important role in HAR is accredited to the activation of human complement (7). Kidneys derived from pigs transgenically expressing human

complement regulatory proteins, such as CD46, CD55, or CD59, survived for up to three months in baboon recipients (106-108).

To overcome cell-mediated xenograft rejection, MHC class I molecules to inactivate human NK cells, and the human FasL to induce apoptosis in activated human T and NK cells, were expressed on pEC (74, 75, 109, 110) (see also results section 5.4.). *In vitro*, such cells were partly protected from cell-mediated xenograft rejection, whereas the physiological relevance still needs to be confirmed by *in vivo* experiments. Recently, pEC derived from HLA-E/β2-microglobulin-transgenic pigs showed partial protection against human NK cell lysis (see results section 5.5.). Another approach, with the aim of immunological tolerance, is the induction of a mixed haematopoietic chimerism (111). Here, the organ recipient receives donor bone marrow prior to organ transplantation to induce central tolerance via thymic presentation of the foreign Ag. Nevertheless, using this method, rejection of the haematopoietic stem cells as well as graft-versus-host reactions, need to be prevented by immunosuppressive strategies. Finally, the transplantation of thymic tissues might also be a possibility to induce tolerance over species barrier (112). An overview on possible strategies to prevent xenograft rejection is shown in table 1.

Hyperacute Rejection	
Complement	Application of soluble complement regulatory proteins (CRP)
	mAb against complement proteins
	Overexpression of CRP on pEC
	Dextran sulfate
Xenoreactive NAb	Immunoabsorption
	Plasmapheresis
	Blocking of anti-αGAL by the administration of soluble αGAL
	Removal of αGAL molecules on pig organs
Acute Vascular Rejection	
Modulation of thrombogenesis	e.g. expression of CD39
Inhibition of Macrophages	
Inhibition of EC activation	e.g. with a mutant NF-κB system
Induction of accommodation	e.g. by the expression of anti-apoptotic genes
Tolerance induction	e.g. by the induction of a mixed haematopoietic chimerism
Inhibition of NK-mediated rejection	Inhibition of NK recruitment to pEC (blocking of chemotaxis, adhesion, and transmigration)
	Inhibition of ADCC (same approaches as described above for xenoreactive NAb)
	Inhibition of NK activation by the transgenic expression of MHC class I molecules on pEC
	Inhibition of NK activation by the elimination of ligands for activating NK receptors
	Elimination of activated Fas-positive NK cells by the transgenic expression of FasL

Table 1: Strategies to prevent xenograft rejection.

3.2. Natural Killer Cells

NK cells are a heterogeneous population of large granular bone marrow-derived lymphocytes that are able to lyse target cells and secrete cytokines, such as interferon (IFN) -γ , and tumor necrosis factor (TNF) -α. They comprise approximately 5-15% of PBMC. As a part of the innate immune system, they do not need any prior sensitization (113). A primary physiological role of NK cells is to provide early defense against pathogenic organisms during the initial response period while the adaptive immune system is being activated (114). Although NK cells respond to a variety of microorganisms, including bacteria and protozoa, they are particularly important in viral infections. NK cells are identified by the surface markers CD16 (FcγRIII) and CD56 (NCAM), and by the lack of T- and B-cell receptors (71). 90% of human peripheral blood NK cells are $CD56^{dim}CD16^{+}$, whereas the other 10% are $CD56^{bright}CD16^{-}$. While the former exhibit high natural cytotoxicity, the latter produce large amounts of cytokines (115). Under physiologic conditions, NK cytotoxicity is accurately regulated in order to lyse only transformed or infected cells (71). Two major checkpoints control target cell susceptibility to NK cytotoxicity: (i) the expression of ligands for various NK activating receptors and (ii) the presence of MHC class I molecules interacting with inhibitory NK receptors. As a result of direct ligand interaction, the inhibitory receptors that bind HLA class I molecules including killer immunoglobulin-like receptors (KIR), Ig-like transcript (ILT) 2 and the CD94/NKG2 family (116), prevent NK cell activation and killing, providing the molecular basis for Kärre's "missing-self" hypothesis (117, 118) (Figure 4). On the other hand, NK cells also express activation receptors, such as NKp30, NKp44, and NKp46 (119), collectively named natural cytotoxicity receptors (NCR), and NKG2D (120), that recognize target cell ligands and can trigger perforin-dependent cytotoxicity. Consequently, NK cytotoxicity occurs if stimulatory signals outweigh inhibitory signals derived from a potential target cell.

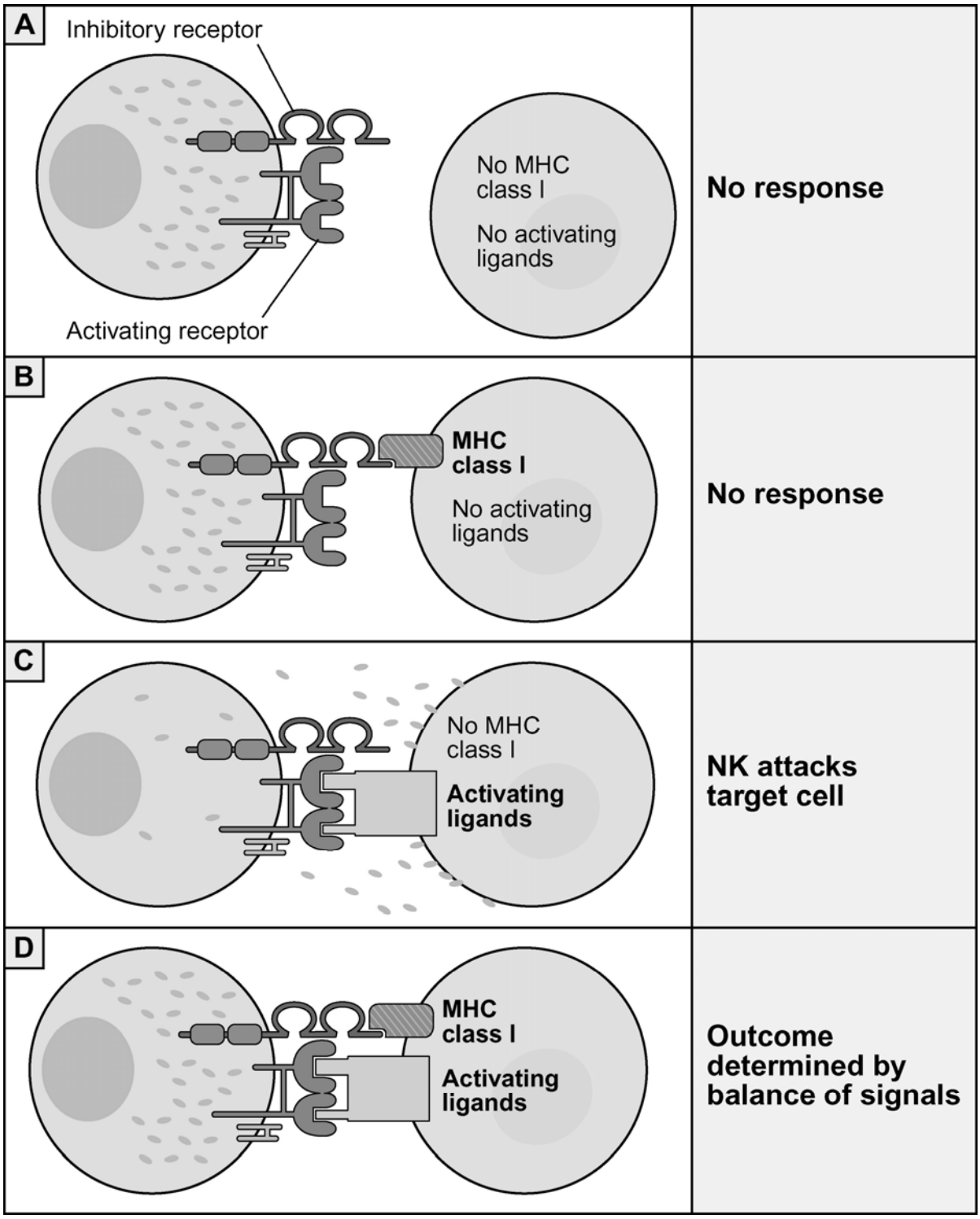

Figure 4: Missing-self hypothesis (117). Schematic picture of possible outcomes after interaction of human NK cells and potential target cells. The amount of activating and inhibitory receptors on NK cells and the amount of corresponding ligands on target cells determine the extent of the NK cell response.

3.2.1. NK Inhibitory Receptors

All of the well-defined inhibitory NK receptors possess one or more copies of the consensus sequence Ile/Val/Leu/Ser-x-Tyr-xx-Leu/Val, where x denotes any amino acid (aa), in their cytoplasmic domains (121). This consensus sequence is called

immunoreceptor tyrosine-based inhibitory motif (ITIM) (122). Upon ligand binding, the tyrosine residue within the ITIM is phosporylated by a Src family kinase and phosphatases are recruited through their SH2-domains. These phosphatases damp or prevent NK cell effector functions, *i.e.* cytotoxicity and cytokine production.

NK receptors that recognize “classical” and “nonclassical” MHC class I molecules have been identified, including the rodent Ly49 receptors, human KIR, Ig-like transcripts (ILT or leukocyte Ig-like receptors, LIR), and conserved CD94/NKG2 receptor family (123) (Table 2). A molecular explanation for the missing-self hypothesis was provided first by the demonstration by Yokohama and colleagues (124) which demonstrated that the subset of NK cells expressing Ly49A receptor were preferentially unable to kill tumor cells expressing H-2D^d. This receptor is the prototypic member of a small gene family that encodes type II transmembrane-anchored glycoproteins expressed on a subset of NK cells and memory T cells. Most of these genes encode ITIM-bearing inhibitory receptors, whereas others are activating receptors without ITIM (125). Detailed insights into NK cell recognition was provided by the analysis of structures of inhibitory Ly49 and H-2 complexes (126). The homodimeric Ly49A receptor binds to H-2D^d at two distinct sites, one of which involves the α1 and α2 domains of MHC class I, whereas the second interaction site spans the α1, α2, α3 domains and β2-microglobulin (β2m).

Humans do not have *Ly49* genes, but an NK receptor system with all of the same general features has evolved to provide the same functions in primates: the human *KIR* gene family. These genes are expressed by subsets of NK cells (127, 128), $\gamma\delta$TCR+ T cells, and memory/effector $\alpha\beta$TCR+ T cells (129). KIR evolved from the Ig superfamily, whereas Ly49 proteins are C-type lectins in structure. KIR receptors are type I transmembrane glycoproteins with two Ig-like domains (designated KIR2D) or three Ig-like domains (designated KIR3D) in the extracellular region (130). Their cytoplasmic domains are variable in length: some receptors possess long (L) cytoplasmic domains with one or two ITIM sequences, and other receptors have short (S) cytoplasmic domains without ITIM. They recognize intact HLA class I trimers, composed of heavy chain, β2m, and peptide, of different type (HLA-A, -B, and -C). Although residues at position seven and eight in the peptide have been implicated as the most important, KIR receptors do not distinguish self from nonself peptides (131). Binding of inhibitory KIR to their MHC class I ligands on potential

target cells results in suppression of cytotoxicity and cytokine secretion by KIR-bearing NK and T cells.

The *CD94* and *NKG2* gene family is present in the genome of humans, rat, and mouse (132). Unlike the *Ly49* and *KIR* genes, the CD94 and NKG2 genes have limited polymorphism, and the minor allelic variants have not been shown to affect the function of these receptors (133). They encode type II transmembrane proteins of the C-type lectin family. CD94 is expressed on cell surface either as a disulfide-linked homodimer or as a disulfide-linked heterodimer with NKG2A, functioning as an inhibitory receptor bearing an ITIM in its cytoplasmic domain, or with NKG2C, serving as an activating receptor (134). CD94/NKG2 receptors are expressed on most NK cells and on several T cell subsets (135). Unlike the KIR and Ly49 receptors that are stably maintained once expressed, CD94/NKG2 receptors are modulated by cytokines in the environment, such as IL-15, TGF-β, and IL-12. Human CD94/NKG2A and CD94/NKG2C heterodimers bind HLA-E, a monomorphic MHC class I molecule expressed at weak levels on most tissues (136-138). The binding of HLA-E to CD94/NKG2A, and subsequent negative signaling, is highly dependent on the nature of the peptide bound to HLA-E and the HLA class I signal sequence-derived peptides are optimal in this regards (136, 139).

Receptor	Ligand	Superfamily
KIR2DL1	HLA-C group 2	Ig-like superfamily
KIR2DL2	HLA-C group 1	Ig-like superfamily
KIR2DL3	HLA-C group 1	Ig-like superfamily
KIR3DL1	HLA-Bw4	Ig-like superfamily
KIR3DL2	HLA-A	Ig-like superfamily
ILT2/LIR1	HLA-G	Ig-like superfamily
CD94-NKG2A/B	HLA-E	C-type lectin

Table 2: NK inhibitory receptors

3.2.1.1. NK Inhibitory Receptors in Xenotransplantation

The susceptibility of pEC to human NK cytotoxicity may be explained by the failure of SLA class I molecules to interact with human NK inhibitory receptors. In line with this, several studies demonstrated that transgenic expression of HLA-Cw3 and HLA-G on pEC provides partial protection from human NK cytotoxicity (74, 75, 79). However,

polymorphic HLA-C expressed on pig cells triggers allorecognition by T cells, which can be prevented by genetical modification of HLA-C (76, 79). Conversely, the wide expression of HLA-E-specific inhibitory receptors of the CD94/NKG2 family on IL-2-activated NK cells proposes HLA-E as an attractive candidate to prevent NK cell-mediated damage in xenotransplants by transgenic expression. Using this concept, conflicting results were obtained so far (77, 78, 140). Although not rigorously examined, human β2m may also be required for maximal cell-surface expression of HLA-E in pig cells. Generating pigs transgenic for HLA-E, the final goal of this approach to protect porcine grafts from human NK cytotoxicity, would therefore require the addition of three human genes (HLA-E heavy chain, human β2m, and a gene encoding for an HLA-E binding peptide) in order to ensure stable HLA-E cell-surface expression. To circumvent this technically difficult and tedious obstacle, a single chain trimer (SCT) of HLA-E has been constructed and expressed in pig cells (141), leading to a significant reduction of NK-mediated cytotoxicity (see results section 5.4.). Furthermore, pEC derived from HLA-E/β2m-transgenic pigs were significantly less susceptible against NK-mediated cytotoxicity *in vitro* (see results section 5.5.).

3.2.2. NK Activating Receptors

Activating receptors on NK cells include NKp30, NKp44, and NKp46 (119), collectively named NCR, and NKG2D (120) (Figure 5, Table 3). NCR play a major role in NK-mediated killing of tumor cell lines as revealed by mAb-mediated receptor-masking experiments. There is a direct correlation between the surface density of NCR and the ability of NK cells to kill various tumors (142). However, as shown by cytotoxicity assays, human NK cells lyse pEC only through NKG2D and NKp44 receptors, whereas NKp30 and NKp46 appear not to play a role in NK-mediated xenogeneic cytotoxicity (73). Despite considerable efforts, the cellular ligands recognized by NCR are still not defined. However, as revealed by cytolytic assays, NCR ligands are expressed by cells belonging to different histotypes (119). While NKp30 and NKp46 are detected on all NK cells regardless of their activation status, NKp44 is selectively expressed by activated NK cells (143). Other triggering surface molecules expressed by NK cells appear to function primarily as co-receptors (119) because their ability to signal depends on simultaneous co-engagement of a main triggering receptor. This group includes NK-specific receptors such as NKp80 (144),

2B4 (145), NTB-A (146), and receptors that are not unique to NK cells such as CD59, CD2, ICAM, CD69, β1 integrins, and DNAM-1. Apart from the direct cytotoxicity described above, NK cells can also be triggered by ADCC. This mechanism is mediated by CD16 on NK cells interacting with immunoglobulins bound to target cells.

Receptor	Ligand	Superfamily
KIR2DS1	HLA-C group 2	Ig-like superfamily
KIR2DS2	HLA-C group 1	Ig-like superfamily
KIR3DS1	HLA-Bw4	Ig-like superfamily
CD94-NKG2C/E	HLA-E	C-type lectin
NKG2D	MIC-A/-B, ULBP1-4	C-type lectin
NKp30	unknown	Ig-like superfamily
NKp44	unknown	Ig-like superfamily
NKp46	unknown	Ig-like superfamily

Table 3: NK activating receptors

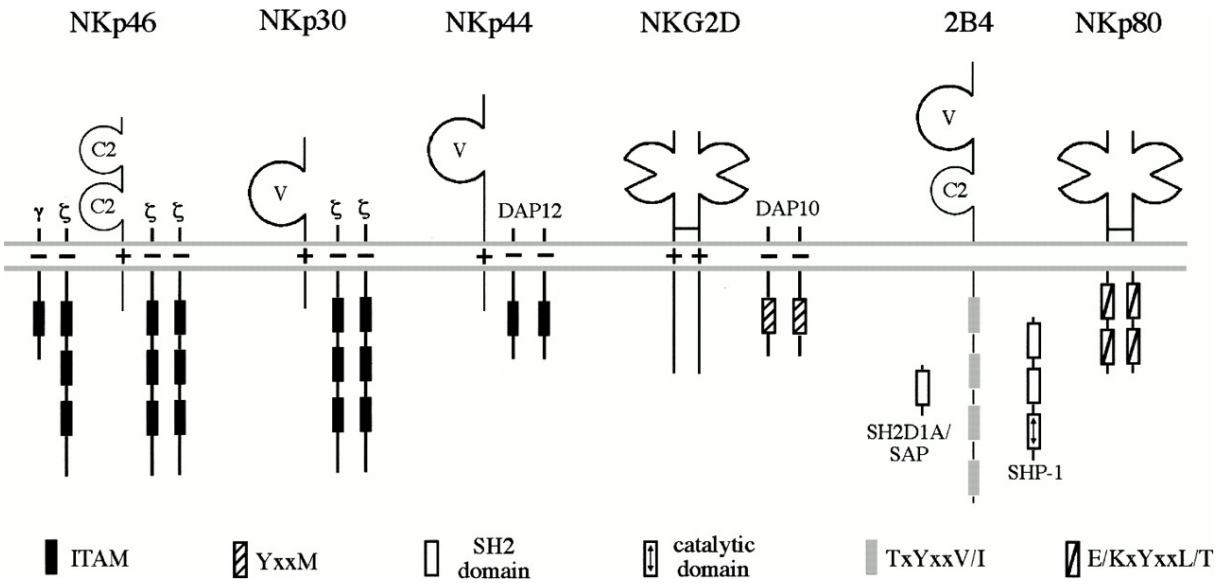

Figure 5: Surface receptors involved in natural cytotoxicity and their association with distinct signal transducing molecules (119). NCR and 2B4 are type I glycoproteins belonging to the Ig-superfamily. NKG2D and NKp80 are dimeric type II glycoproteins belonging to the C-type lectin receptor family.

3.2.2.1. NKG2D and its Ligands

NKG2D, a C-type lectin surface receptor, is a member of the NKG2 family, but only distantly related to the other members of the family and unlike the others, does not

form heterodimers with CD94 (147). It is expressed as a disulfide-linked homodimer on the surface of all mouse and human NK cells and most γδTCR+ T cells (148). The NKG2D expression on NK and T cells can be increased by culture in IL-15 or TNF-α , but is significantly downregulated in the presence of TGF-β. In contrast to the other triggering receptors, the surface expression of NKG2D requires association with DAP10 or KAP10 (149). DAP10 is characterized by the presence of a negatively charged residue in the transmembrane portion and by a Tyr-xx-Met motif (where x denotes any aa) in the cytoplasmic tail that, upon tyrosine phosphorylation, binds to PI3-kinase.

Several ligands of NKG2D on human cells have been identified, namely the stress-inducible UL16-binding proteins (ULBP) (150) or MHC class I chain-related proteins A and B (MICA/B) (151). The ULBPs were identified based on their ability to bind the human cytomegalovirus (CMV) glycoprotein UL16. These are glycosylphosphatidyl-inositol (GPI) -linked proteins, distantly related to members of the HLA class I family possessing α1 and α2 domains, but not an α3 domain (152) and they are unable to present peptides (153). MICA and MICB, on the other hand, possess all three α-domains and are both transmembrane proteins. Like the ULBPs, MICA and MICB do not bind β2m or present peptides (154).

In an attempt to identify the porcine ligands for human NKG2D, molecular cloning and characterization of a porcine homologue of the human ULBP proteins, porcine ULBP1 (pULBP1) has recently been described (155). PULBP1 exhibits 35-52% aa identity to human ULBPs and phylogenetic analyses place pULBP1 evolutionarily close to the bovine ULBP-like genes MHCLA1 and MHCLA2. The pULBP1 aa sequence exhibits a relatively high level of aa conservation at positions predicted to make contact with NKG2D (153). Southern blot analysis suggested that only one pULBP exists in the pig genome (155), which is in sharp contrast to the at least six expressed in humans. However, considering hybridization conditions in these studies, the possibility that other more distantly related porcine ULBP-like genes exist cannot be excluded.

The sequence of porcine MIC2 (pMIC2), a porcine homologue of the human MIC proteins, has been previously reported (156). It is comprised of six exons and the predicted aa sequence displays characteristics similar to those of the human MIC genes, such as four N-glycosylation sites, three of which are apparent counterparts of the human glycosylation sites at position 208, 235 and 263. In addition, several

cysteine residues within the pMIC2 α2 and α3 domains may participate in the formation of disulfide bonds. Similarly to the human MIC genes, a consensus heat shock element, but no iron response element, was found upstream of the porcine exon one. Both pULBP1 and pMIC2 transcripts could be detected in pEC and pMIC2 transcripts were increased following heat shock or retinoic acid treatment (155). But only pULBP1 represents a functional ligand for the human NKG2D (157) (see results section 5.2.).

3.2.2.2. NKp44

NKp44 displays a molecular size of 44 kDa and induces triggering of NK-mediated cytotoxicity upon cross-linking by specific mAb (143). Its expression is restricted to IL-2-activated NK cells, whereas it is absent in fresh peripheral blood NK cells. Therefore, NKp44 can be used as a specific marker for activated human NK cells. Moreover, IL-2-activated NK cells acquire an increased cytolytic activity against NK-susceptible targets, also of swine origin (158). NKp44 is a glycoprotein with a protein backbone that associates with the immunoreceptor tyrosine-based activating motif (ITAM)-bearing DAP12 signal-transducing molecules that become tyrosine phosphorylated upon NKp44 cross-linking (159). Molecular cloning revealed a member of the Ig-superfamily characterized by a single extracellular V-type domain, a positively charged transmembrane domain (containing the aa Lys), and a short cytoplasmic domain without ITAM. The association of Lys with DAP12 molecules that contain a single ITAM in the cytoplasmic portion and are expressed as disulfide-bonded homodimers, goes over negatively charged residues in the transmembrane domain of DAP12. The ligand(s) for NKp44 remain(s) still unidentified, although it was described that viral hemagglutinins expressed on the surface of virus-infected cells are involved in the recognition by NKp44 and NKp46 (160).

3.2.2.3. NKp30 and NKp46

NKp30 and NKp46 were both identified as activating NK receptors by redirected killing assays. They are expressed on all NK cells, irrespective of their state of activation. The molecular size is approximately 30 kDa and 46 kDa, respectively, and they are associated with CD3ζ chains that contain several ITAM (119). Molecular cloning of the cDNAs encoding for NKp30 (161) and NKp46 (162) revealed members of a type I transmembrane glycoprotein belonging to the Ig superfamily. For NKp30,

the extracellular portion is characterized by a single domain of the V-type and by a region rich in hydrophobic aa. NKp46 has two C2-type Ig-like domains in the extracellular portion, followed by a stretch of aa possibly forming a stem connecting the ectodomain to the transmembrane region. The transmembrane regions contain the positively charged aa Arg, probably involved in the association with CD3ζ chains. The cytoplasmic portions do not contain ITAM typically involved in the activation of signal cascades, but NKp30 and NKp46 are coupled to the intracytoplasmic transduction machinery by their association with CD3ζ chains and, in the case of NKp46, also with FcεRIγ, that contains ITAM. Cross-linking of NKp30 and NKp46 by mAb resulted in Ca^{2+} flux, cytotoxicity and cytokine production. Unlike NKG2D, the ligands for NKp30 and NKp46 remain elusive.

4. Aims of the Study

The overall goal of this study was to increase our knowledge of NK cell-mediated xenogeneic rejection mechanisms. Here, I focussed on the identification of factors regulating the cytotoxic potential of human NK cells against pEC. Because NK cells are considered to play a significant role in xenograft rejection, a better understanding of the complex interactions between receptors on human NK cells and their corresponding ligands on pEC might lead to novel strategies to improve xenotransplant survival.

1. NK cells are tightly regulated by inhibitory as well as activating receptors that are expressed on their surface. Inhibitory receptors, such as KIR, recognize MHC class I molecules and prevent NK cells from killing a potential target cell. On the other hand, there are four major activating receptors, namely NKG2D and the NCR NKp30, NKp44, and NKp46, which promote NK cell-mediated cytotoxicity. Their surface density on NK cells correlates with the magnitude of cytolytic activity against NK cell-susceptible target cells. Corresponding ligands to these receptors are expressed on the surface of a variety of normal human cells, but are upregulated on tumour and virus-infected cells. However, this study is the first to investigate the role of activating NK cell receptors in a pig-to-human xenotransplant setting. Furthermore, the general role of NK cell-mediated cytotoxicity against pEC and the phenotype and cytotoxic potential of a panel of NK cell clones were analyzed.

2. Having identified two important activating NK receptors (NKG2D and NKp44) in stage 1 of the project, the next step was to identify their corresponding ligands on porcine cells. One advantage of xenotransplantation is the possibility to genetically manipulate pig organs, with the aim of maximizing transplant survival with an organ-specific approach, and reducing the amount of drugs and therapies necessary to administer to the graft recipient. NKG2D is expressed on all human NK cells regardless of their activation status, whereas NKp44 is only expressed on IL-2-activated human NK cells. Thus, NKG2D may play a more important role in NK cell-mediated xenograft rejection. Porcine ULBP1 and pMIC2 are homologues of the human NKG2D ligands ULBP1-4 and MICA/MICB, respectively. Although pULBP1 and pMIC2 were known to be expressed in pEC, one aim of this study was to

investigate whether these surface molecules also act as functional ligands for human NKG2D. After the identification of pULBP1 as the predominant, if not the only, porcine ligand for human NKG2D, a mAb was generated and used to characterize pULBP1 surface expression levels on pEC after various treatments, such as cytokine stimulation, co-incubation with human serum, or CMV infection.

3. Human NK cell-mediated cytotoxicity against porcine cells may be caused by incompatible cross-species interactions between porcine MHC class I molecules and inhibitory human NK cell receptors. This notion was supported by the finding that expression of specific HLA class I molecules on pEC, such as HLA-E, results in complete protection from cytotoxicity mediated by NK cell clones expressing the respective HLA-specific inhibitory receptor (110). However, only partial protection from cytotoxicity mediated by polyclonal human NK cell populations was observed. Furthermore, it was shown that this protection depends on extrinsic peptide loading. Although not rigorously examined, human β2m may also be required for maximal cell-surface expression of HLA-E in pig cells. The generation of transgenic pigs providing organ grafts resistant to CD94/NKG2A$^+$ human NK cell-mediated cytotoxicity may therefore require transgenic expression of three different human genes (HLA-E heavy chain, human β2m, and a gene encoding for an HLA-E-binding peptide), in order to ensure stable expression of HLA-E on cell surface. To circumvent this technically difficult model, a SCT variant of HLA-E has been constructed and expressed in pig cells (141). This construct was composed of a canonical HLA-E-binding peptide antigen, VMAPRTLIL, the mature human β2m, and the mature HLA-E heavy chain. One aim of this study was to test whether the exogenous expression of HLA-E SCT protects pEC against xenogeneic cytotoxicity mediated by IL-2-activated human NK cells derived from healthy donors. Furthermore, pEC derived from HLA-E/β2m-transgenic pigs were tested for their ability to resist NK cell-mediated cytotoxicity *in vitro*.

5. Results

5.1. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D

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Author contributions:: PF and BGL designed, performed, and analyzed the experiments and wrote the manuscript. BCB contributed to the phenotypical analysis of the NK cell clones. JDS supervised the project and contributed to paper writing.

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Running title: Activating receptors mediating xenogeneic NK cytotoxicity

Key Words: NK Cells, Cell surface molecules, Cytotoxicity, Transplantation

Abbreviations used in this paper: ADCC: antibody-dependent cell-mediated cytotoxicity; αGAL: galactose-alpha-1,3-galactose; KIR: killer immunoglobulin-like receptors; MFIR: geometric mean fluorescence intensity ratio; MICA/MICB: MHC class I chain-related proteins A/B; NCR: natural cytotoxicity receptors; pEC: porcine endothelial cells; ULBP: UL16-binding proteins.

5.1.1. Abstract

Pig-to-human xenotransplantation has been proposed as a means to alleviate the shortage of human organs for transplantation, but cellular rejection remains a hurdle for successful xenograft survival. NK cells have been implicated in xenograft rejection and are tightly regulated by activating and inhibitory receptors recognizing ligands on potential target cells. The aim of the present study was to analyze the role of activating NK receptors including NKp30, NKp44, NKp46, and NKG2D in human xenogeneic NK cytotoxicity against porcine endothelial cells (pEC). ^{51}Cr-release and antibody blocking assays were performed using freshly isolated, IL-2-activated polyclonal NK cell populations as well as a panel of NK clones. Freshly isolated NK cells are NKp44 negative and lysed pEC exclusively in an NKG2D-dependent fashion. In contrast, the lysis of pEC mediated by activated human NK cells depended on both NKp44 and NKG2D, since a complete protection of pEC was achieved only by simultaneous blocking of these activating NK receptors. Using a panel of NK clones a highly significant correlation between anti-pig NK cytotoxicity and NKp44 expression levels was revealed. Other triggering receptors such as NKp30 and NKp46 were not involved in xenogeneic NK cytotoxicity. Finally, antibody-dependent cell-mediated cytotoxicity (ADCC) of pEC mediated by human NK cells in the presence of xenoreactive Ab was not affected by blocking of activating NK receptors. In conclusion, strategies aimed to inhibit interactions between NKp44 and NKG2D on human NK cells and so far unknown ligands on pEC may prevent direct NK responses against xenografts but not xenogeneic ADCC.

5.1.2. Introduction

The clinical use of porcine organs to alleviate the current shortage of human organs in transplantation medicine is impeded by the occurrence of several types of vigorous rejection mechanisms that lead to rapid graft failure (4, 6). Recently, advances in the prevention of hyperacute rejection in preclinical models using genetically engineered pigs suggest the importance of both coagulation disorders and cellular immunity in human anti-pig xenogeneic responses (103, 104). Despite the fact that prolonged survival of these xenografts has been achieved without specifically inhibiting NK cells, they might play an important role in endothelial injury and delayed rejection of porcine xenografts. This hypothesis is supported by the finding that *in vitro* NK cells activate porcine endothelium upon direct contact and act as a potent source for pro-

inflammatory cytokines such as interferon-γ (48, 53). On the other hand, NK cell activation depends, amongst others, on T cell-derived IL-2 stimulation, an interaction between the innate and adaptive immune system that might be interrupted by conventional immunosuppressive protocols used in preclinical models for xenotransplantation. Human NK cells have further been demonstrated to adhere to and lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive Ab, by Ab-dependent cell-mediated cytotoxicity (ADCC) (46, 54). In addition, pig organs perfused with human blood *ex vivo* are predominantly infiltrated by NK cells (57, 59) and NK cells are present in histological samples of graft rejection in concordant and discordant rodent and preclinical pig-to-baboon models (60-62). Thus, although the potential role of NK cells is still controversial, strategies to inhibit both direct NK cytotoxicity and ADCC against porcine cells might facilitate successful clinical xenotransplantation.

Under physiologic conditions NK cytotoxicity is accurately regulated in order to lyse only transformed or infected cells (71). Two major checkpoints control target cell susceptibility to NK cytotoxicity: (i) the expression of ligands for various NK activating receptors and (ii) the presence of MHC class I molecules interacting with inhibitory NK receptors. The killing signals transduced by activation receptors are balanced by several groups of inhibitory receptors that bind HLA class I molecules including killer immunoglobulin-like receptors (KIR), ILT2 and the CD94/NKG2 family (116). Consequently, NK cytotoxicity occurs when NK cells encounter ligands for activating receptors on potential target cells that, in addition, have lost or downregulated MHC class I expression. Porcine endothelial cells (pEC) are susceptible to human NK-mediated lysis possibly due to the inability of their MHC class I molecules to signal through human NK cell inhibitory receptors (72).

Theoretically, xenogeneic NK cytotoxicity could be avoided either by expressing HLA class I molecules on porcine cells or by blocking the function of activating receptors. Indeed, we and others have previously demonstrated that the expression of human MHC class I molecules including HLA-B27, -Cw3, -E, and -G on porcine cells provided partial protection from lysis mediated by polyclonal human NK cells (74-79). However, complete inhibition of NK cytotoxicity has not been achieved by transgenic HLA class I expression since the corresponding NK inhibitory receptors for each HLA

molecule were expressed only on NK subpopulations. Moreover, protection from ADCC by HLA class I expression has been demonstrated only for NK clones, but not for polyclonal NK cells (76, 80).

Activating receptors on NK cells include NKp30, NKp44, and NKp46 (119), collectively named natural cytotoxicity receptors (NCR), and NKG2D (120). NCR play a major role in NK-mediated killing of tumor cell lines as revealed by mAb-mediated receptor-masking experiments. Their surface density on NK cells correlates with the magnitude of cytolytic activity against NK-susceptible target cells. Despite considerable efforts, the cellular ligands recognized by NCR are still not defined. However, as revealed by cytolytic assays, NCR ligands are expressed by cells belonging to different histotypes (119). While NKp30 and NKp46 are detected on all NK cells regardless of their activation status, NKp44 is selectively expressed by activated NK cells (143). In contrast to NCR, several ligands of NKG2D have been identified, including the stress-inducible MHC class I chain-related proteins A (MICA) and B (MICB) or UL16-binding proteins (ULBP) (150). Other triggering surface molecules expressed by NK cells appear to function primarily as co-receptors (119), because their ability to signal depends on simultaneous co-engagement of a main triggering receptor. This group includes NK-specific receptors such as NKp80, 2B4, NTB-A, and receptors that are not unique to NK cells such as CD59, CD2, ICAM, CD69, β1 integrins, and DNAM-1. Apart from the direct cytotoxicity described above, NK cells can also be triggered by ADCC. This mechanism is mediated by Fc□RIII receptors (CD16) on NK cells interacting with immunoglobulins bound to target cells.

The aim of the present study was to explore the hypothesis that human NK cytotoxicity against porcine cells might be overcome by blocking activating NK receptors. We demonstrate that human NK cytotoxicity against pEC depends exclusively on NKp44 and NKG2D signals.

5.1.3. Material and Methods

Cells

Two SV40-immortalized pEC lines, the aortic PEDSV.15 and the bone-marrow-derived microvascular 2A2 cell lines, were established and characterized in our laboratory (163). A primary aortic pEC (PAEC) was isolated using a protocol

previously described (163) and cultivated in S199 (Gibco, Basel, Switzerland) supplemented with 15% FCS (PAA Laboratories, Lucerne, Switzerland), non-essential amino acids (100x) (Invitrogen, Basel, Switzerland), 1% penicillin/streptomycin (Gibco), 20mM HEPES (Invitrogen), 50 μg/ml ECGS (BD Biosciences, Allschwil, Switzerland), and 100 μg/ml heparin (Sigma, Buchs, Switzerland). The human melanoma cell line Mel-15 (a kind gift of M. Urosevic, University Hospital Zürich), the immortalized porcine lymphoblastoid cell line 13271.10 (a kind gift of G. Waneck (Massachusetts General Hospital, Boston, MA) (76, 164) and the human lymphoblastoid cell line 721.221 (ATCC, Molsheim Cedex, France) (165) were cultured in RPMI (Invitrogen) supplemented with 12.5% FCS. Isolation of PBMCs from healthy blood donors, purification of NK cells, and generation of monoclonal and polyclonal human NK cell populations have been described previously (74). After isolation, the purity of NK cells was routinely > 95%, the cells were either used directly or activated by culture in AIM-V medium (Invitrogen) supplemented with 10% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, essential amino acids (50x), non-essential amino acids (100x), 1% penicillin/streptomycin, 20 mM HEPES (all Invitrogen), and 300 U/ml of human IL-2 (Chiron, Emeryville, CA).

Flow cytometry

Surface expression of NKp30, NKp44, NKp46, and NKG2D on human NK cells was analyzed on a FACScan (Becton Dickinson, Basel, Switzerland) by indirect immunofluorescence using the following primary mouse mAb: Z25 (anti-NKp30, IgG1, Beckman Coulter/Immunotech, Marseille, France), Z231 (anti-NKp44, IgG1, Beckman Coulter/Immunotech), BAB281 (anti-NKp46, IgG1, Beckman Coulter/Immunotech), 149810 (IgG1, anti-NKG2D, R&D Systems, Abingdom, UK), supernatants of AZ20 (IgG1, anti-NKp30), Z231, BAB281 and ON72 (IgG1, anti-NKG2D) were a kind gift of A. Moretta, University of Genova. FITC-conjugated goat anti-mouse IgG Ab (Chemicon International, Dietikon, Switzerland) was used as a secondary reagent. Human NK cells were resuspended at 2 to $5x10^5$ cells per tube in staining buffer (HANKS, 0.1% BSA) and incubated for 30 min at 4°C with saturating Ab concentrations. Phenotypic analysis of NK cells was carried out by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-

B159 (anti-CD56) mAb (all from Pharmingen, Allschwil, Switzerland). An irrelevant, isotype-matched control mAb (MOPC21, mouse IgG1, Sigma) was used as control and propidium iodide gating to exclude dead cells in all experiments. To compare the levels of surface expression, the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of staining with the mAb of interest with the mean fluorescence intensity of the control mAb.

Cytotoxicity assays

The cytotoxic activity of freshly isolated and IL-2-activated monoclonal and polyclonal human NK cells was tested in two or four hours ^{51}Cr-release assays in serum-free AIM-V medium as described previously (166). Briefly, labeled target cells were added to triplicate samples of serial twofold dilutions of NK cells in round-bottom 96-well plates. Four E:T ratios ranging from 40:1 to 2.5:1 were determined in each experiment. For blocking studies, NK cells were preincubated for 30 min at 4°C with 10 μg/ml mAb (for all figures except figure 4A) or 10□l hybridoma supernatant (for figure 4A) either independently or in combinations. Monoclonal Ab were also present during the coincubation of target and effector cells at a concentration of 5 μg/ml. To determine ADCC, ^{51}Cr-release assays were performed in the presence of 10% decomplemented (heat-inactivated) human serum. Human sera were obtained from healthy adult volunteers. Decomplementation by heat-inactivation was carried out at 56°C for 30 min. Samples were stored at 4°C for short periods or aliquoted and stored at -20°C. After incubation for 2 or 4 h at 37°C, the assays were stopped, ^{51}Cr-release was analyzed on a gamma counter and the percentage of specific lysis was calculated.

5.1.4. Results

Expression of natural cytotoxicity receptors and NKG2D on human NK cells

To comprehensively evaluate the role of activating receptors involved in human NK cell-mediated lysis of pEC, it was necessary to carefully determine the cell surface expression of NCR and NKG2D on the NK cells used for functional studies under our conditions. Freshly isolated NK cells were NKp30, NKp46, and NKG2D positive, but NKp44 negative (Fig. 1A), whereas IL-2-activated NK cells were also positive for NKp44 (Fig. 1B). The MFIR of NKG2D and NKp30 were higher on activated NK cells,

whereas the MFIR of NKp46 was higher on freshly isolated NK cells. We also analyzed the surface expression of activating NK receptors 2 to 3 weeks following limiting dilution cloning on NK clones cultured in the presence of IL-2. MFIR values equal or greater than 1.5 were considered to be positive. In close agreement to the analyses of polyclonal NK populations, all NK clones analyzed (n=77) were NKG2D positive (mean MFIR of 13 ± 6), 97% were also NKp44 positive (mean MFIR of 9 ± 6), 91% were NKp30 positive (mean MFIR of 3 ± 1) and 71% were NKp46 positive (mean MFIR of 2 ± 0.6). In a repeated flow cytometry analysis after additional four weeks of culture no significant changes in the expression level of NKp30, NKp44, and NKG2D were observed, whereas all NK clones were now NKp46 positive. However, these results were obtained from a limited number of NK clones (n=7), due to their restricted life-time in culture. The NKp44 and NKG2D expression pattern of the polyclonal NK populations from which the clones were generated was similar: 97% of the cells were NKG2D positive (MFIR of 19) and 50% NKp44 positive (MFIR of 3.5). In contrast, only a minor fraction of polyclonal NK populations was NKp30 (8%, MFIR of 1.6) or NKp46 (2%, MFIR of 2.5) positive. Flow cytometry analysis of the activating NK receptors was always repeated at the day of the cytotoxicity assay.

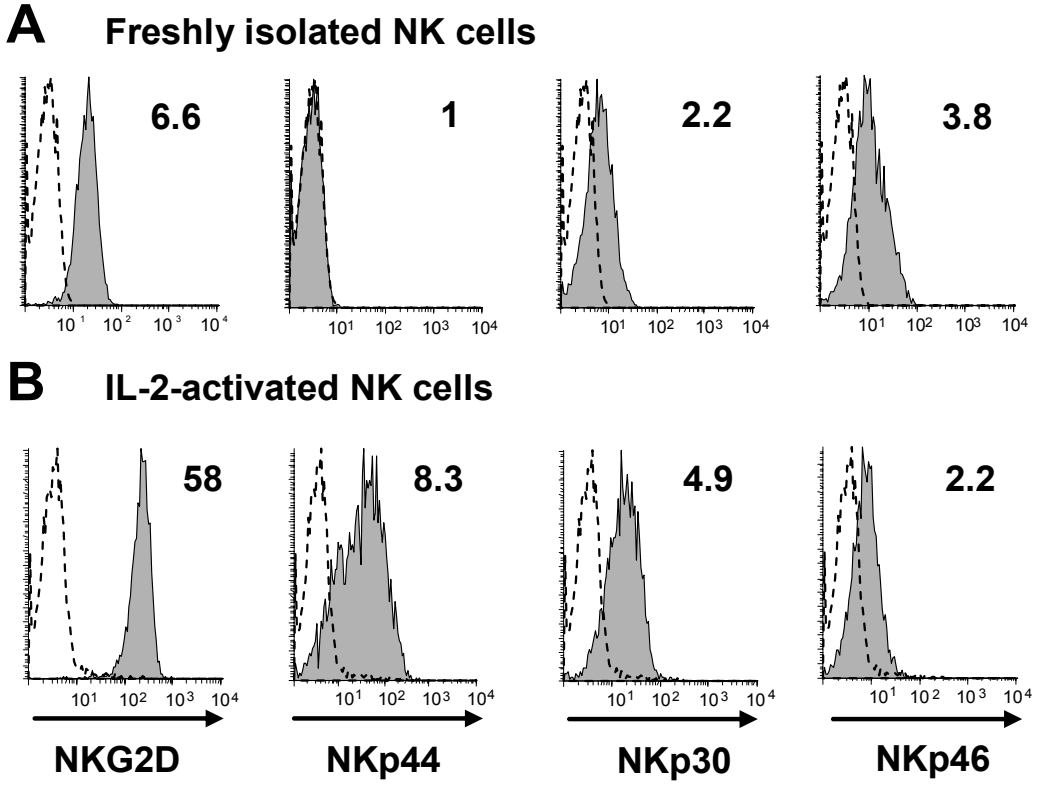

Figure 1: Cell surface expression of NCR and NKG2D. Freshly isolated (A) and IL-2-activated (B) NK cells were analyzed by flow cytometry using either anti-NKp30 , -NKp44, -NKp46, and -NKG2D

mAb (filled histograms) or an isotype-matched control mAb (empty histograms, dashed lines). Numbers indicate MFIR.

Cytotoxicity of freshly isolated and IL-2-activated NK cells against immortalized porcine endothelial cells

Freshly isolated NK cells and polyclonal NK populations generated in the presence of IL-2 were tested for their ability to lyse the immortalized pEC lines PEDSV.15 and 2A2. A substantial difference in NK cytotoxicity against PEDSV.15 mediated by freshly isolated versus IL-2-activated NK cells at an E:T ratio of 20:1 was evident (21 ± 9% versus 48 ± 22% median specific lysis, n=10 and 37, respectively, Fig. 2A). The lysis of 2A2 cells by activated NK cells was comparable (43 ± 21% median specific lysis, n=38), whereas lysis of 2A2 cells by freshly isolated NK cells was clearly lower (8 ± 5%, n=9), as shown in Figure 2B.

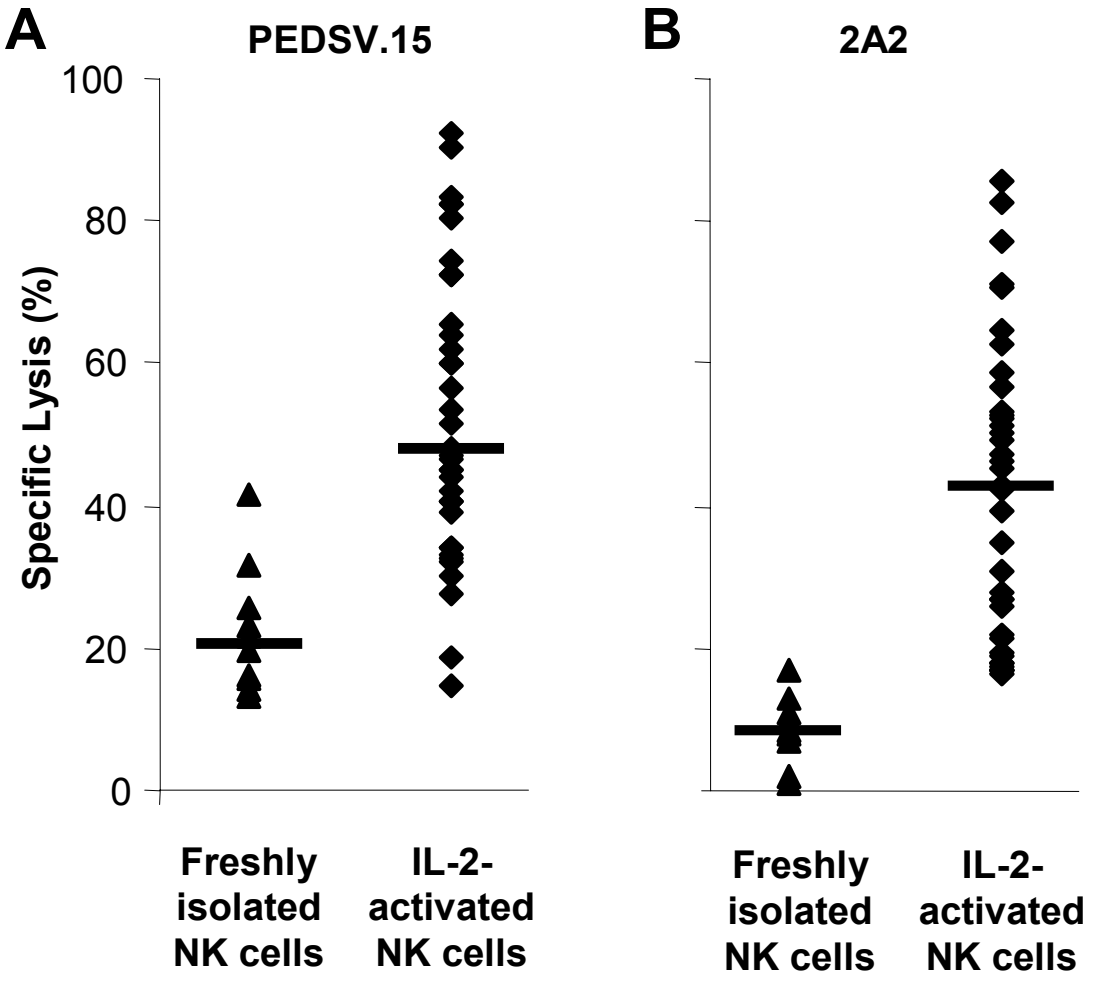

Figure 2: Cytotoxicity mediated by freshly isolated or IL-2-activated human NK cells against PEDSV.15 and 2A2 target cells. A summary of the cytotoxic activity of freshly isolated (triangles) and IL-2-activated (diamonds) NK cells against PEDSV.15 (A) and 2A2 (B) cells is shown. Cytotoxicity was

measured in 4 h ^{51}Cr-release assays at an E:T ratio of 20:1 and is expressed as percentage of specific lysis. Each triangle/diamond represents one independent experiment, the horizontal lines represent the median value of lysis.

The variability among different donors in the ability of their freshly isolated NK cells to lyse pEC was remarkable. This finding is illustrated in Figure 2. Overall, freshly isolated NK cells displayed a cytolytic activity ranging between 3 and 54% at an E:T ratio of 40:1. Importantly, freshly isolated NK cells displaying a weak cytotoxicity against porcine targets were still able to efficiently lyse the MHC class I-deficient human target cell line 721.221 (data not shown). Blocking of NKp30, NKp44 and NKp46 independently or in combination did not lead to a reduced cytotoxicity of freshly isolated NK cells against PEDSV.15 (Fig. 3). Regardless of the individual killing efficiency of different donors, blocking of NKG2D was always able to reduce xenogeneic cytotoxicity of freshly isolated NK cells significantly without further reduction of cytotoxicity by additional blocking of NKp30 and NKp46 (Fig. 3B).

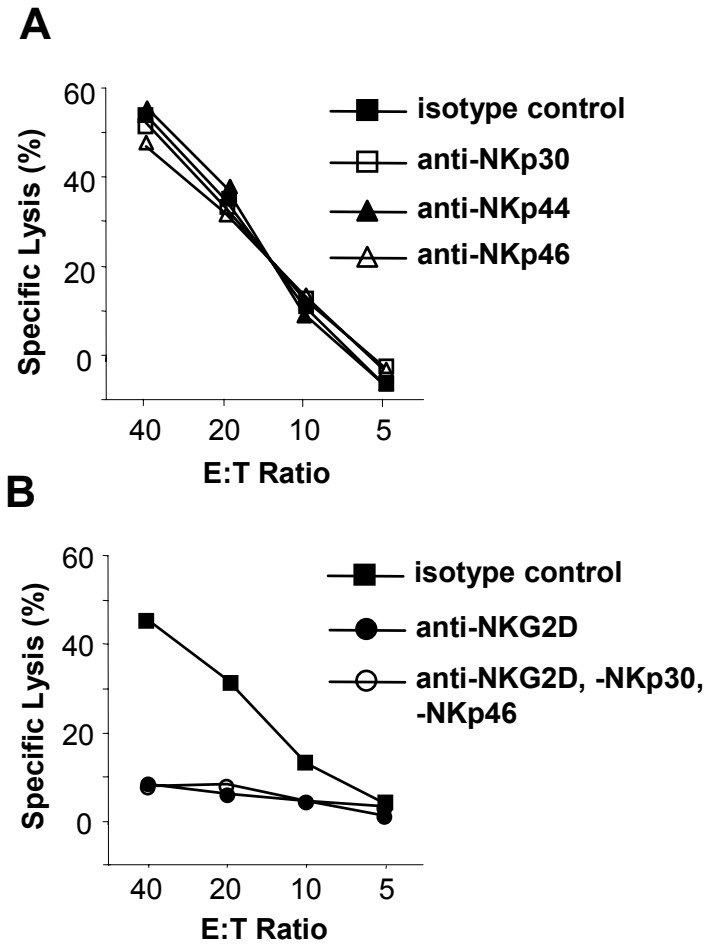

Figure 3: NKG2D triggers cytotoxicity of freshly isolated NK cells against PEDSV.15. Freshly isolated NK cells purified from different donors were analyzed for the cytolytic activity against PEDSV.15 in the presence of the following mAb: IgG_1 isotype-control, anti-NKp30, anti-NKp44, anti-NKp46, anti-NKG2D, or anti-NKG2D/-NKp30/-NKp46 mAb in combination. All results are expressed

as percentage of specific lysis and were obtained at four different E:T ratios. Results are representative for one out of four (A) and one out of ten (B) independent experiments performed with NK cells purified from five different donors.

In contrast, the cytolytic activity of IL-2-activated human polyclonal NK cells against PEDSV.15 depended on both NKG2D and NKp44, but not on NKp30 or NKp46. A partial inhibition of xenogeneic NK cytotoxicity was observed by mAb-mediated masking of either NKp44 (44% inhibition) or NKG2D (56% inhibition) while isotype-matched control mAb as well as blocking of NKp30 or NKp46 had no effect (Fig. 4A). Neither did a combination of anti-NKp44 mAb with either anti-NKp30 or anti-NKp46 mAb enhance the inhibitory effect of anti-NKp44 (data not shown).. As a positive control for the activity of the anti-NKp30 and -NKp46 mAb to inhibit NK killing we used the human melanoma cell line MEL-15. NK-mediated lysis of MEL-15 was clearly reduced in blocking assays using NKp30 and NKp46 mAb alone or in combination (data not shown). Nearly complete blocking of activated NK-cell cytotoxicity against PEDSV.15 was achieved by simultaneous blocking of NKp44 and NKG2D receptors (Fig. 4B and C). No difference with regard to the blocking efficiency was observed comparing 2 h to 4 h ^{51}Cr-release assays (Fig. 4B and C), we only observed a higher specific lysis when co-incubating target and effector cells for longer time periods. The cytotoxic response of freshly isolated and IL-2-activated NK cells against pEC did not correlate with NKG2D receptor surface densities that were similar between different donors (data not shown).

Altogether, these data indicate that NKG2D plays a pivotal role in xenogeneic NK cytotoxicity mediated by both freshly isolated and IL-2-activated human NK cells whereas NKp44 triggers lysis mediated by IL-2-activated human NK cells. In contrast, NKp30 and NKp46 do not play a role in human NK cytotoxicity against pEC.

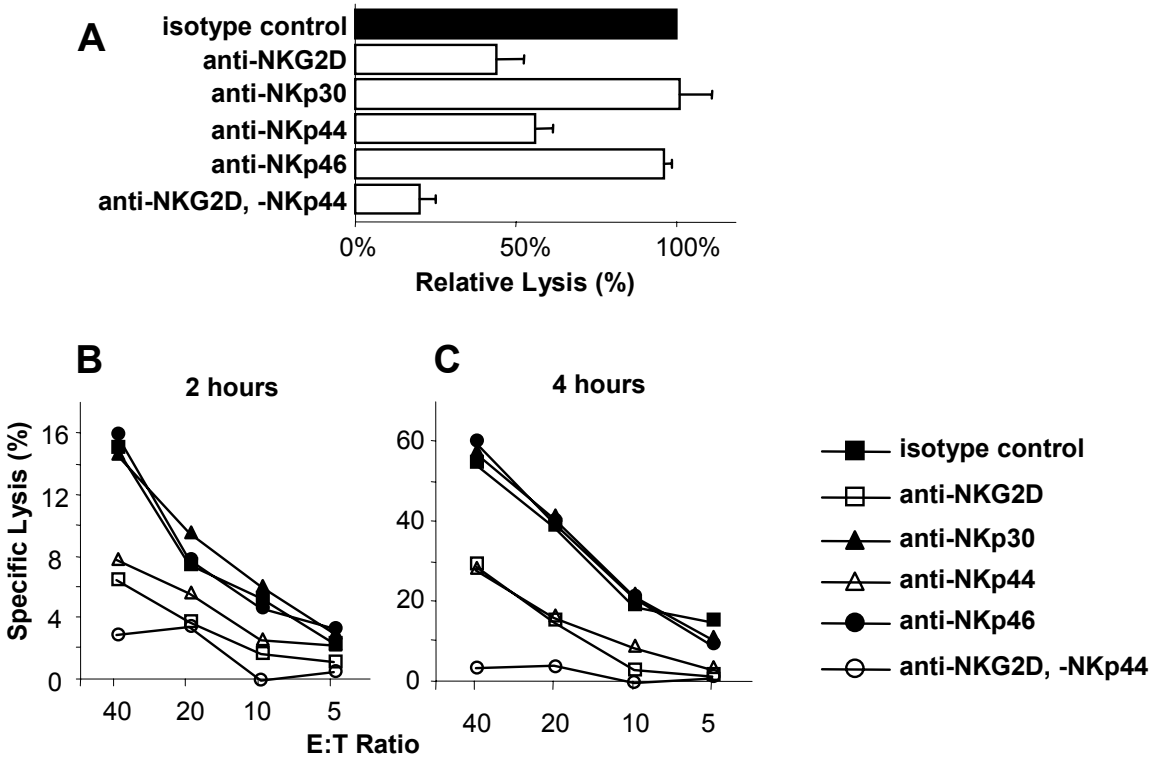

Figure 4: NKp44 and NKG2D but not NKp30 and NKp46 receptors trigger cytotoxicity of NK cells against PEDSV.15 cells. The cytotoxic activity of IL-2-activated NK cells was tested against PEDSV.15 cells in two and four h ^{51}Cr-release assays in the presence of different combinations of the following mAb: IgG_1 isotype-control, anti-NKG2D, anti-NKp30, anti-NKp44, anti-NKp46, and anti-NKG2D/-NKp44 in combination.(A) shows a summary of four independent experiments where cytotoxicity is expressed as percentage of relative lysis of PEDSV.15 cells in the presence of the indicated mAb as compared to the respective intra-assay control (lysis with isotype-control mAb, index=100). The mean relative cytotoxicity was calculated at four different E:T ratios (40:1 to 5:1), error bars indicate SEM. (B and C) show representative experiments for one out of five (B) and one out of 15 (C) independent experiments performed with NK cells purified from ten different donors.

Xenogeneic cytotoxicity of NK clones against immortalized porcine aortic endothelial cells correlates with NKp44 expression levels

To further analyze the role of NCR and NKG2D in NK-mediated xenogeneic lysis of PEDSV.15 a panel of NK clones was generated. NK clones expressing different levels of NKG2D and NCR provide a unique tool to study the role of these activating receptors. The potential of NK clones to lyse PEDSV.15 cells was tested in the absence or presence of mAb against NCR and NKG2D. Lysis of PEDSV.15 mediated by an NK clone expressing high levels of NKp30 (MFIR of 9) and NKp46 (MFIR of 6) was markedly reduced (93% inhibition) only by blocking with NKp44- and NKG2D-specific mAb, but not by blocking with NKp30- and NKp46-specific mAb (Fig. 5A). The combined use of anti-NKp44, -NKG2D, -NKp30, and -NKp46 mAb did not further enhance the inhibitory effect of anti-NKp44/NKG2D mAb, providing

additional evidence that NKp30 and NKp46 receptors do not recognize any ligands on PEDSV.15.

In addition, ten different NKG2D-positive NK clones with MFIR for NKp44 expression ranging from 3 to 26, were analyzed for their capacity to lyse PEDSV.15. In the absence of NKG2D-mediated activation, i.e. in the presence of saturating concentrations of anti-NKG2D mAb, NK cytotoxicity was proportional to the expression level of NKp44. Thus, as shown in Figure 5B, PEDSV.15 cells were efficiently killed by NKp44^{bright} NK clones while they were partially resistant to cytotoxicity mediated by NKp44^{dull} NK clones. In contrast, clonal NK cytotoxicity did not correlate with NKp30 and NKp46 cell surface densities.

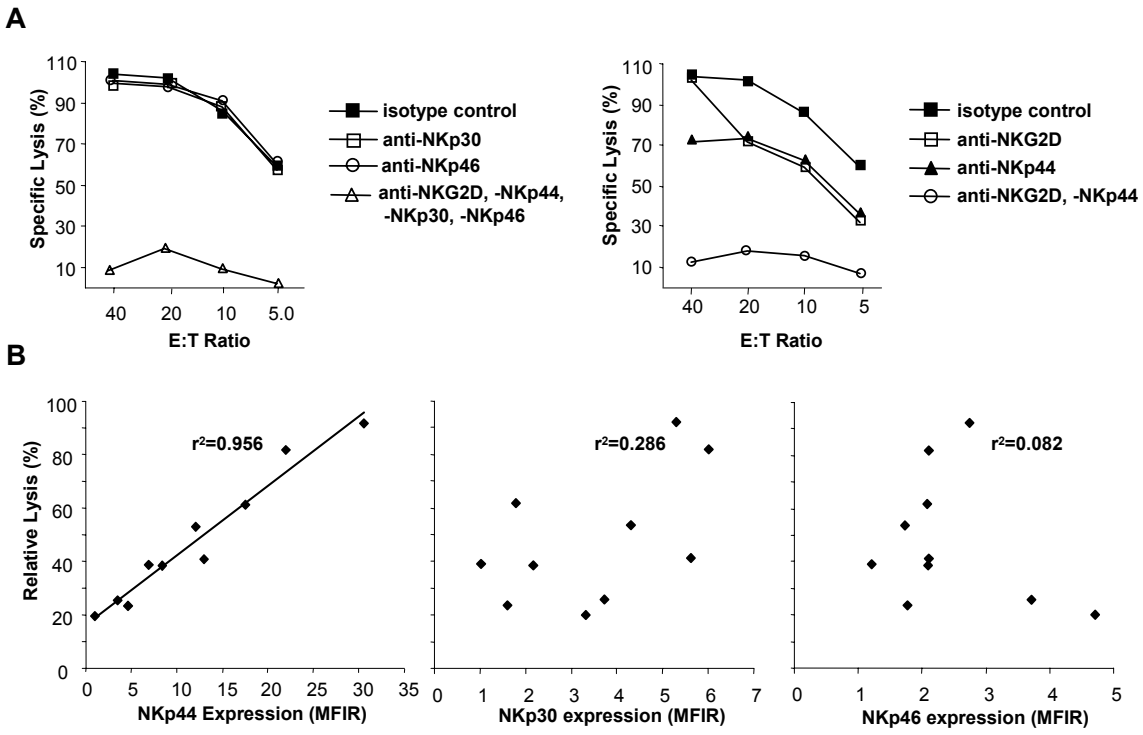

Figure 5. Cytotoxicity of NK clones against PEDSV.15 cells is proportional to NKp44 expression and independent from NKp30 and NKp46. (A) The cytotoxic activity of an NK clone expressing high levels of NCR and NKG2D molecules was tested against PEDSV.15 in the presence of the following mAb: IgG_1 isotype-control, anti-NKG2D, anti-NKp30, anti-NKp44, anti-NKp46, anti-NKG2D/-NKp44 and anti-NKG2D/-NKp44/-NKp30/-NKp46 in combination. This experiment is representative for one out of two independent experiments. (B) shows cytotoxicity of different NK clones in relation to their NKp44, NKp30 and NKp46 expression. Cytotoxicity is expressed as percentage of relative lysis of PEDSV.15 cells in the presence of anti-NKG2D mAb as compared to the respective intra-assay control (lysis without mAb, index=100). The correlation coefficient (r^2) is 0.956 for NKp44, 0.286 for NKp30 and 0.082 for NKp46. NKp44, NKp30 and NKp46 expression is indicated as MFIR. Each square represents a value from a single NK clone.

Lysis of primary aortic and immortalized bone marrow-derived porcine endothelial cells as well as porcine lymphoblastoid cells depends on NKp44 and NKG2D

The finding that NKp44 and NKG2D are responsible for IL-2-activated xenogeneic NK lysis of PEDSV.15 cells raised the question about the role of these receptors in the lysis of other porcine target cells. Thus, NK cytotoxicity against primary porcine aortic derived EC (PAEC) (Fig. 6A) and immortalized bone marrow-derived pEC (2A2) (Fig. 6B) was analyzed. Both PAEC and 2A2 were protected from IL-2-activated human NK cytotoxicity in the presence of anti-NKp44 and -NKG2D mAb, whereas isotype-matched and anti-HLA class I mAb had no effect. NK cytotoxicity against 2A2 seemed to depend predominantly on NKp44 as compared to NKG2D, whereas PEDSV.15 cells were mainly killed through NKG2D (Fig. 4). In line, we observed that 2A2 cells were clearly less susceptible than PEDSV.15 to NK cytotoxicity mediated by freshly isolated NKp44 negative NK cells (Fig. 2B). Taken together, these results show that NKp44 and NKG2D function as unique receptors involved in human NK-mediated cytotoxicity against pEC independent of the respective NK donor. In order to expand our study to cell types other than endothelial cells, xenogeneic human NK cytotoxicity against porcine lymphoblastoid cells was tested. In parallel to pEC, blocking of NKG2D and NKp44 inhibited the lysis of lymphoblastoid cells (Fig. 6C), whereas NKp30 and NKp46 had no effect (data not shown). In contrast to pEC, blocking with NKG2D and NKp44 mAb was not sufficient to prevent the lysis of lymphoblastoid cells indicating that other activating receptors are involved in the lysis of non-endothelial hematopoietic target cells.

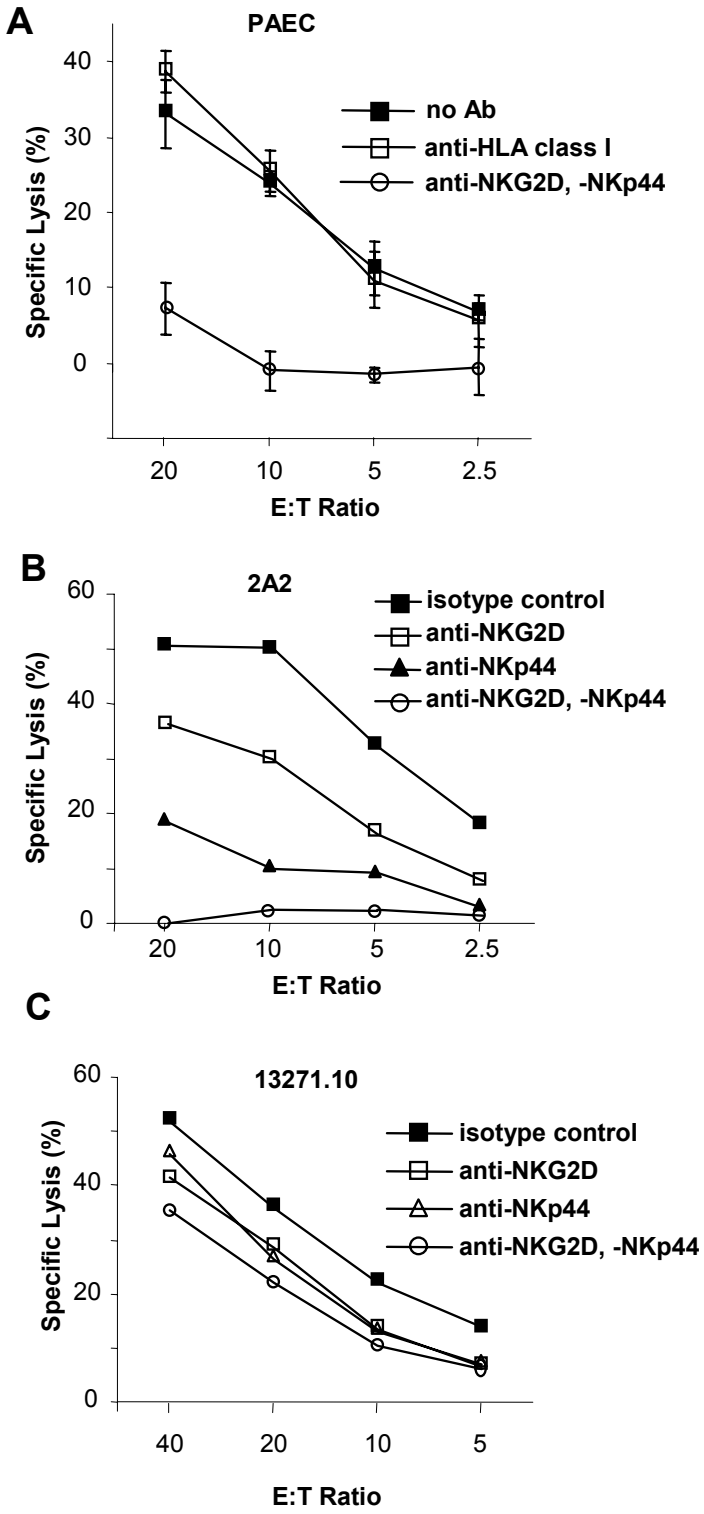

Figure 6: NKp44 and NKG2D mediate NK cytotoxicity against primary porcine aortic endothelial cells (PAEC), the bone marrow-derived 2A2 cell line and the lymphoblastoid cell line 13271.10. The cytotoxic activity of IL-2-activated NK cells was tested against PAEC (A), 2A2 cells (B) and 13271.10 (C) in a 4 h ^{51}Cr-release assay in the presence of the following mAb: IgG_1 isotype-control, anti-HLA class I, anti-NKG2D, anti-NKp44, and anti-NKG2D/-NKp44 in combination . (A) shows the mean specific lysis of three independent cytotoxicity assays using PAEC as targets, error bars indicate SEM. The experiments shown in (B) and (C) are representative for one out of three (B) and one out of six (C) independent experiments.

Xenogeneic ADCC is not prevented by interfering with NKG2D activation

ADCC of human NK cells against pEC represents a very efficient mechanism of lysis. While the involvement of CD16 in ADCC is well documented the role of other activating receptors has currently not been investigated. Therefore, the contribution of NCR and NKG2D in xenogeneic NK-mediated ADCC against pEC was analyzed. NK cytotoxicity mediated by freshly isolated NK cells against PEDSV.15 cells was evaluated in the presence of heat-inactivated human serum containing saturating amounts of xenoreactive natural Ab. The addition of human serum clearly increased the lysis of PEDSV.15 by freshly isolated NK cells, demonstrating a strong NK cell-mediated ADCC. Blocking with NCR- and NKG2D-specific mAb in ^{51}Cr-release assays revealed no inhibitory effect on NK cell-mediated ADCC against PEDSV.15 cells (Fig. 7). These findings indicate that activation signals transmitted by CD16 are sufficient to induce efficient lysis of pEC and do not require additional activation signals through NKG2D, NKp30 or NKp46.

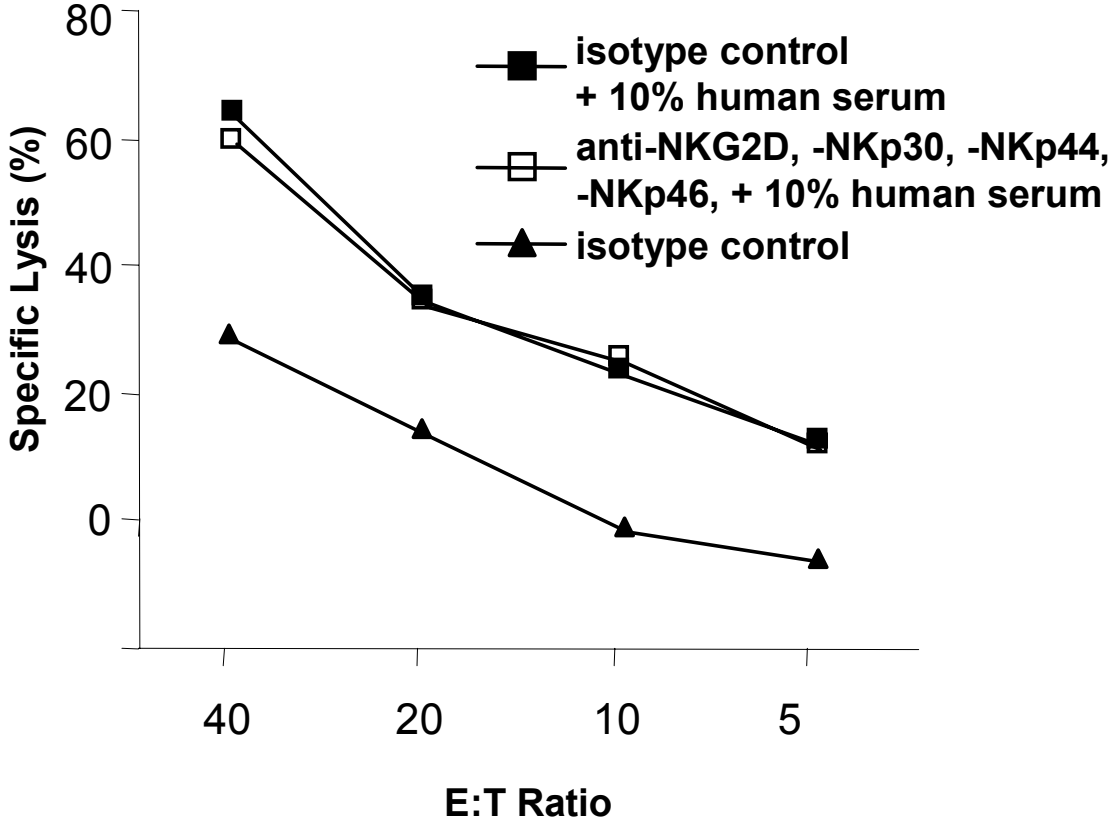

Figure 7: ADCC is independent of NKG2D and NCR signalling. The cytotoxicity of freshly isolated NK cells was tested against PEDSV.15 in the presence (triangles) or absence (squares) of 10% decomplemented human serum in 4 h ^{51}Cr-release assays. The following blocking mAb were used as indicated: IgG_1 isotype-control, anti-NKG2D, anti-NKp30, anti-NKp44, and anti-NKp46. The data shown are representative for one out of three independent experiments performed with NK cells purified from totally eight different donors.

5.1.5. Discussion

The generation of galactose-alpha-1,3-galactose (alphaGal) knockout pigs has overcome the barrier of anti-Gal mediated rejection in pig-to-nonhuman primate xenotransplantation. Therefore, strategies to reduce other rejection mechanisms need to be explored. These may include natural or elicited non-Gal-specific xenoreactive Ab and cellular immunity mediated by direct NK cell responses, all of which lead to porcine endothelial activation, injury and eventually to graft dysfunction. Human NK cytotoxicity against pEC is readily observed *in vitro*, whether or not it contributes to xenograft rejection *in vivo* remains unknown. Nevertheless, recognition of xenogeneic pEC by human NK cells *in vitro* leads to activation and injury of pEC by several mechanisms. Taking the limitations of *in vitro* studies into careful consideration, NK cytotoxicity studies may represent relevant NK cell responses *in vivo*. Similarly, the level and specificity of allogeneic cytotoxic T cell responses *in vitro* reproduce *in vivo* graft rejection which also does not necessarily involve cytotoxic functions. Here, we further analyzed the molecular mechanisms leading to NK cell activation and cytotoxicity in response to porcine endothelium, the first immunological target following vascularized pig-to-human xenotransplantation. Human NK cytotoxicity against porcine target cells requires cross-species compatibility between human NK activating receptors and their respective porcine ligands. At the same time it reflects the absence of sufficient interactions between NK inhibitory receptors and MHC class I molecules across the species barrier. Whereas human NK cells are inhibited only weakly, if at all, by swine MHC class I molecules (167), human activating NK receptor interactions with porcine ligands have not been investigated so far.

The data presented here provides clear evidence that NKG2D and NKp44 function as triggering receptors involved in direct human NK cytotoxicity against pEC, while NKp30 and NKp46 do not play a role. NKG2D was the only activating receptor involved in pEC lysis mediated by freshly isolated NK cells. Intriguingly, the significant variability among donors in the response of freshly isolated NK cells against pEC did not correlate with NKG2D receptor surface densities that were similar between different donors. Therefore, the reason for this variability might depend on the different efficiency of the respective cytotoxic effector pathways such as the perforin-granzyme release that is important in xenogeneic NK cytotoxicity

(168). Indeed, it has been shown that NK cell culture conditions, in particular IL-2 and IL-15 supplements, up-regulate the expression levels of molecules involved in the perforin-granzyme cytolytic pathway (169). Moreover, the cytolytic potential of individual freshly isolated NK populations might depend on the immunologic condition of the donor since NK cells collected at different time points from the same donor differed in their ability to kill pEC without apparent differences in NKG2D expression (data not shown). The importance of NKp44 for NK cytotoxicity mediated by activated human NK cells against pEC was clearly supported by (i) antibody blocking studies, (ii) the correlation of lysis with NKp44 expression levels on NK clones, and (iii) the association of poor xenogeneic NK cytotoxicity against 2A2 cells with the absence of NKp44 on freshly isolated NK cells. Since it was not addressed in the present study, we can only speculate on the role of the numerous additional co-receptors involved in human NK cytotoxicity. Their triggering function appears to depend on the simultaneous engagement of the main activating receptors (119).

The involvement of NKp44 and NKG2D in NK-mediated lysis of pEC indicates the presence and recognition of porcine homologues of human NKp44 and NKG2D ligands on pEC or of "unique" ligands in pigs without apparent human homologues. Compatibility of NK activating receptors and their respective ligands has also been described in other species combinations. In fact, the putative ligand for the human NKp46 receptor is responsible for xenogeneic NK-mediated lysis of murine cells (170). The notion that NCR and NKG2D receptors are, at least partially, conserved between humans and other species is clearly different from the divergent evolution that has been documented for the MHC class I-specific inhibitory receptors (171-173). Allelic specificity of human KIR for HLA class I molecules suggests a co-evolution of inhibitory KIR with the respective MHC genes. This might explain why human KIR have a low affinity for MHC class I molecules of unrelated species (174). In contrast, it is conceivable that NCR and NKG2D as well as their ligands have not been subjected to the pressure that caused the evolution of MHC genes and their receptors. The porcine NCR and NKG2D ligands are unknown, however, comparison of porcine and human genomic sequences suggests the presence of one ULBP gene and one MIC-A/-B-like gene (MIC2) in the pig genome (175, 176). NKG2D ligands are generally poorly expressed by normal cells but are up-regulated in transformed, infected, and stressed cells in both mice and humans (120). These findings are

compatible with the supposed expression of porcine NKG2D ligands on transformed pEC lines like PEDSV.15 and 2A2 but are in contrast with the involvement of NKG2D in NK-mediated lysis of primary pEC. However, stress generated by isolation and cell culture conditions could be responsible for NKG2D ligand expression on primary pEC and subsequent susceptibility to xenogeneic NK lysis. Similarly, it is easy to predict that triggers of inflammation, ischemia reperfusion injury, and rejection mechanisms following xenotransplantation might also allow the expression of porcine ligands of human NKG2D. This hypothesis is supported by the finding that human kidney allografts undergoing both acute and chronic rejection episodes have been shown to express polymorphic MIC molecules which may even induce allospecific Ab (177, 178). Future studies are needed to identify the porcine ligands for NKG2D and NKp44, thereby it will be of interest whether only two or more ligands are involved in this interspecies NK recognition. Beside cytokine secretion, endothelial cell activation and direct NK cytotoxicity, human NK cells are also able to kill porcine cells via ADCC. As reported previously, the lack of alphaGal on porcine cells strongly reduced NK-mediated ADCC, whereas binding and direct cytotoxicity of human NK cells were not inhibited (44, 179). The fact that ADCC was not influenced by blocking NCR and NKG2D indicated that the two mechanisms of human NK cytotoxicity against porcine target cells are independent.

Emerging evidence over the past few years indicated that the expression of HLA class I molecules in porcine tissues did not provide complete protection from direct xenogeneic human anti-pig NK cytotoxicity through inhibitory receptors. In the present paper we demonstrate that the abrogation of human NKp44 and NKG2D interactions with their porcine ligands was able to completely prevent direct human NK cytotoxicity against pEC. Thus, the identification and elimination of porcine ligands of NKp44 and NKG2D might have important implications by representing a complementary approach to protect porcine xenografts from human NK cell responses including direct xenogeneic NK cytotoxicity.

5.1.6. Acknowledgments

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kindly providing the MEL-15 cell line. M.D. Crew (University of Arkansas for Medical Sciences, Little Rock, Arkansas) and M.K.J. Schneider (University Hospital Zürich, Switzerland) are acknowledged for careful reading of the manuscript and helpful comments.

5.2. Porcine UL16-binding protein 1 expressed on the surface of endothelial cells triggers human NK cytotoxicity through NKG2D

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Author contributions: BGL designed, performed, and analyzed all the experiments and wrote the manuscript. CGB and MDC cloned the vectors for the fusion proteins, generated the stable transfectant HEK293/pULBP1, and contributed to paper writing. JDS supervised the project and contributed to paper writing.

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Running title: Porcine ligand for human NKG2D

Key words: Natural Killer Cells, Cell Surface Molecules, Transplantation

[3]Abbreviations used in this paper: ADCC: antibody-dependent cell-mediated cytotoxicity; MICA/B: MHC class I chain-related protein A/B; MFIR: geometric mean fluorescence intensity ratio; NCR: natural cytotoxicity receptors; PAEC: porcine aortic endothelial cells; pEC: porcine endothelial cells; pMIC2: porcine MHC class I chain-related protein 2; pULBP1: porcine UL16-binding protein1; shRNA: short hairpin RNA; siRNA: short interfering RNA; SLA: swine leukocyte antigen; ULBP: UL16-binding protein.

5.2.1. Abstract

Cellular rejection mechanisms including NK cells remain a hurdle for successful pig-to-human xenotransplantation. Human anti-pig NK cytotoxicity depends on the activating receptor NKG2D. Porcine UL-16 binding protein 1 (pULBP1) and porcine MHC class I chain-related protein 2 (pMIC2) are homologues of the human NKG2D ligands ULBP 1-4 and MICA and B, respectively. Although transcribed in porcine endothelial cells (pEC) it is not known whether pULBP1 and pMIC2 act as functional ligands for human NKG2D. Here, surface protein expression of pULBP1 was demonstrated by flow cytometry using a novel pULBP1-specific polyclonal Ab and by cellular ELISA using NKG2D-Fc fusion protein. Reciprocally, pULBP1-Fc bound to primary human NK cells, whereas pMIC2-Fc did not. Transient and stable downregulation of pULBP1 mRNA in pEC using short interfering RNA (siRNA) oligonucleotide duplexes and short hairpin RNA (shRNA), respectively, resulted in a partial inhibition of xenogeneic NK cytotoxicity through NKG2D in ^{51}Cr-release assays. In contrast, downregulation of pMIC2 mRNA did not inhibit NK cytotoxicity. Human NK cytotoxicity against pEC mediated by freshly isolated or IL-2-activated NK cells through NKG2D was completely blocked using anti-pULBP1 polyclonal Ab. In conclusion, this study suggests that pULBP1 is the predominant, if not only, functional porcine ligand for human NKG2D. Thus, the elimination of pULBP1 on porcine tissues represents an attractive target to protect porcine xenografts from human NK cytotoxicity.

5.2.2. Introduction

Organ shortage is a severe problem in transplantation medicine, therefore pig-to-human xenotransplantation has become an important field of research to overcome this hurdle (4). Currently, insufficient long-term graft function prevents the successful clinical application of xenotransplantation (6). Nevertheless, advances in preventing hyperacute rejection in preclinical non-human primate models using genetically engineered pigs as organ source indicate that all coagulation disorders, antibodies to non-Gal antigens, and cellular immunity play a role in xenogeneic responses (103, 104, 180). There are also several lines of evidence that NK cells may be a factor in delayed rejection of porcine xenografts (46). Pig organs perfused with human blood *ex vivo* are infiltrated by NK cells (57, 59) and NK cells are present in histological samples of graft rejection in concordant and discordant rodent and pig-to-baboon

models (60-62). In addition, *in vitro* NK cells lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive Ab, by Ab-dependent cell-mediated cytotoxicity (ADCC) (54). In contrast to hematopoietic stem cell transplantation, the involvement of NK cells in rejecting organ grafts was thought to be of minor importance until recently. However, several new findings support the notion that NK cells also participate in the immune response against solid organ allo- and xenografts (47, 181). Thus, implementation of strategies to inhibit NK cytotoxicity and other yet to be defined NK cell responses such as cytokine release and enhancement of adoptive immune responses may promote both successful clinical xenotransplantation and allotransplantation.

NK cells are tightly regulated through signals mediated by inhibiting and activating receptors expressed on their cell-surface (71). Many of the inhibitory NK receptors recognize MHC class I molecules and therefore allow NK cells to discriminate between normal self, non-self and altered cells (116). Porcine endothelial cells (pEC) are susceptible to human NK cell-mediated lysis possibly due to the inability of swine leukocyte antigen (SLA) class I molecules to signal through human NK inhibitory receptors (72, 74). On the other hand, the activation of human NK cells by potential ligands expressed on pEC may be equally, or more, important. Activating receptors on NK cells include NKp30, NKp44, and NKp46 (119), collectively named natural cytotoxicity receptors (NCR), and the C-type lectin receptor NKG2D (120). While NKp30 and NKp46 are detected on all NK cells regardless of their activation status, NKp44 is selectively expressed by activated NK cells (143). There is a direct correlation between the surface density of NCR and the ability of NK cells to lyse various tumor cells (142), but the cellular ligands recognized by NCR remain unidentified. As recently reported, human NK cytotoxicity against pEC is mediated primarily through NKG2D and NKp44, whereas NKp30 and NKp46 are not involved (81).

NKG2D is constitutively expressed on NK, NKT, and CD8-positive T cells as a homodimeric receptor associated with the transmembrane adaptor molecule DAP10 (148). Over the past few years several regulation mechanism of surface expression have been reported (120, 182). Cellular ligands of NKG2D include the stress-inducible MHC class I chain-related proteins A/B (MICA/B) (151) and UL16-binding

proteins (ULBP) (150). The latter were identified based on their ability to bind the human CMV glycoprotein UL16. Although these GPI-linked proteins are distantly related to members of the HLA class I family possessing α1 and α2, but not α3 domains (152), they are unable to present peptides (153). On the other hand, both MICA and MICB are transmembrane proteins and possess all three α-domains. Similar to ULBP, MICA and MICB do not bind β2-microglobulin or present peptides (154).

Blocking of human NK cytotoxicity against porcine cells with anti-NKG2D mAb provided indirect evidence for cross-species interactions between human NKG2D and one or several different porcine ligands (81). However, little is known about the nature and function of these putative ligands. The porcine NKG2D gene has been cloned and revealed a 66% sequence identity with human NKG2D, transcripts are expressed in PBL, NK cells, macrophages, and monocytes (183). The sequence of porcine MIC2 (pMIC2), a homologue of human MIC proteins, has also been reported previously (156). It is comprised of six exons and the predicted amino acid sequence displays characteristics similar to those of the human MIC genes, such as four N-glycosylation sites, three of which are apparent counterparts of the human glycosylation sites at position 208, 235 and 263. In addition, several cysteine residues within the pMIC2 α2 and α3 domains may participate in the formation of disulfide bonds. Similarly to the human MIC genes, a consensus heat shock element, but no iron response element, was found upstream of the porcine exon one. Only recently, porcine ULBP1 (pULBP1) was cloned and characterized as a homologue of human ULBP (155). Phylogenetic analyses place pULBP1 evolutionarily close to the bovine ULBP-like genes MHCLA1 and MHCLA2. It exhibits 35-52% amino acid identity to human ULBP including a relatively high level of conservation at positions predicted to make contact with human NKG2D (153). Southern blot analysis suggested that only one pULBP exists in the pig genome, which is in sharp contrast to the much higher number of ULBP genes, at least six, that were described in humans. However, considering hybridization conditions in these studies (155), the possibility that other more distantly related porcine ULBP-like genes exist can not be fully excluded.

Since both pULBP1 and pMIC2 transcripts were detected in a pEC (155) they represent potential ligands for human NKG2D. Therefore, the aim of the present study was to test whether pULBP1 and pMIC2 can act as functional ligands for human NKG2D resulting in xenogeneic human anti-pig endothelial cell NK cytotoxicity. Several lines of evidence are presented which suggest that pMIC2 is ineffective in binding to NKG2D, and that pULBP1 is the predominant, if not only, porcine ligand for human NKG2D.

5.2.3. Material and Methods

Cells

The SV40-immortalized aortic pEC line PEDSV.15 was established and characterized in our laboratory (163). PAEC were isolated from a normal pig following standard procedures (166) and cultured in DMEM (Invitrogen AG, Basel, Switzerland) supplemented with 10% FCS (PAA Laboratories, Luzern, Switzerland), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x), essential amino acids (50x), and 20 mM HEPES (all Invitrogen). Isolation of PBMCs from healthy blood donors, purification of NK cells, and generation of polyclonal human NK cell populations have been described previously (74). Isolated NK cells with a purity of routinely >95% were either used directly or activated by culture in AIM-V medium (Invitrogen) supplemented with 10% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, essential amino acids (50x), non-essential amino acids (100x), 1% penicillin/streptomycin (Invitrogen), 20 mM HEPES, and 300 U/ml of human IL-2 (Chiron, Emeryville, CA). The immortalized human NK line NK92 (a kind gift of C. Falk, University of Munich, Germany) was cultured in RPMI 1640 (Invitrogen) supplemented with 15% FCS (PAA Laboratories), 5% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% penicillin/streptomycin. The human embryonic kidney cell line HEK293 (a kind gift of J. Wittwer, University Hospital Zürich, Switzerland) was cultured in the same medium as PAEC.

Fusion protein production

The vector encoding a pULBP1-Fc fusion protein (amino acids 1–219, seven amino acid linker, Fc of human IgG1) under control of the CMV immediate-early promoter was constructed by standard techniques and was stably expressed in HEK293 cells

selected with 1 mg/ml G418 (Invitrogen). After limiting dilution cloning, a clone secreting high amounts of fusion protein was selected and expanded. The pULBP1-Fc fusion protein was purified using a protein A column (Amersham Biosciences, Freiburg, Germany) and dialysed against PBS. Protein concentration was determined photospectrometrically. The purity and specificity of the fusion protein pULBP1-Fc was confirmed by western blot and coomassie staining showing one specific band. A gene encoding a pMIC2-Fc fusion protein was similarly constructed and was transiently expressed in HEK293 cells and pMIC2-Fc was purified from culture medium using a protein A column.

Polyclonal Ab production

Two rabbits were immunized with pULBP1-Fc by i.v. injection of 150 μg protein in CFA, followed by three booster immunizations with 100 μg protein in IFA (every four weeks). Four weeks after the last boost, sera were collected, purified using a protein A column and dialysed against PBS. One of the rabbits elicited a specific immune response. Preimmune sera of the immunized rabbits were collected for control experiments.

Flow cytometry

Surface expression of NKG2D on human NK cells, and expression of pULBP1 on pEC was analyzed on a FACScan (Becton Dickinson, Basel, Switzerland) by indirect immunofluorescence. After resuspension of $1x10^5$ cells per tube in staining buffer (HANKS, 0.1% BSA) cells were incubated for 30 min at 4°C with saturating amounts of Ab. As primary Ab the mouse mAb 149810 (IgG1, anti-NKG2D, R&D Systems, Abingdom, UK) and a polyclonal rabbit anti-pULBP1 (obtained in our laboratory, see Results), respectively, were used. As secondary reagents FITC-conjugated goat anti-mouse IgG Ab (Chemicon International, Dietikon, Switzerland) and FITC- or PE-conjugated goat anti-rabbit IgG Ab (Sigma, Buchs, Switzerland) were used. Phenotypic analysis of NK cells was carried out by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (all from Pharmingen, Allschwil, Switzerland). An irrelevant, isotype-matched control mAb (MOPC21, mouse IgG1, Sigma) was used as control for mAb stainings, and normal rabbit IgG (R&D Systems) or preimmune sera were used as control for the polyclonal rabbit Ab. To exclude dead cells, propidium iodide gating was performed

in all experiments. To compare the levels of surface expression, the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of each sample with the mean fluorescence intensity of the control Ab.

Cytotoxicity assays

The cytotoxic activity of freshly isolated and IL-2 activated human NK cells was tested in 4 h ^{51}Cr-release assays in serum-free AIM-V medium as described previously (166). Briefly, labeled target cells were added to triplicate samples of serial twofold dilutions of NK cells in round-bottom 96-well plates. Four E:T ratios ranging from 40:1 to 5:1 were used in each experiment. For blocking studies, NK cells were preincubated for 30 min at 4°C with 10 μg/ml of the following mAb: MOPC21, 3G8 (mouse IgG1, anti-CD16, Immunotech, Nyon, Switzerland), and 149810. MAb were also present during the coincubation of target and effector cells at a concentration of 5 □g/ml. A saturating amount of 10 μl of the polyclonal anti-pULBP1 Ab as well as normal rabbit IgG were used for blocking studies. After incubation for 4 h at 37°C, the assays were stopped, ^{51}Cr-release was analyzed on a gamma counter and the percentage of specific lysis was calculated.

Short interfering RNA oligonucleotides and short hairpin RNA vectors

The following targeting siRNA oligonucleotide duplexes were purchased from Proligo (Paris, France): pMIC2 (5'-ACAGCUUCACAGAGAGAACA-3'), pULBP1 (5'-CCACAUCGAUUCAGACAAUAU-3'). Transfection of PEDSV.15 cells was performed using X-tremeGene siRNA transfection reagent (Roche Applied Science, Switzerland). A transfection efficiency of >95% was determined by FACS analysis 8 hours post transfection using a 3'-fluorescein-labelled control siRNA (5'-CUACCAAUACUUCATT(Fluo)-3'). The shRNA expression vector pRNAT-H1.1 (Genscript, NJ, USA) was used to construct the following vectors: pULBP1-targeting vector pRNAT-H1.1-U2 (encoding the shRNA ACUUU-GUACCUUUCACCUUCUCUUGAUAUCCGGAGAAGGUGAAAGGUACAAAG) and pMIC2-targeting vector pRNAT-H1.1-M3 (encoding the shRNA AUUGGUCUCUUU-ACGCCAUGUCUUGAUAUCCGGACAUGGCGUAAAGAGACCAAU). PEDSV.15 cells were Ca_2PO_4 co-transfected with each of these vectors and pCDNA3.1-hygromycin (Invitrogen) and selected with 200 μg/ml hygromycin (Invitrogen).

RT-PCR and quantitative RT-PCR

RNA was isolated using Trizol (Invitrogen), and the concentration was determined photospectrometrically. The RNA quality was verified by agarose gel electrophoresis. Reverse transcription was achieved using AMV Reverse Transcriptase (Promega, Wallisellen, Switzerland) following the manufacturers protocol. The following primers were used in PCR: β2-microglobulin-specific (5'-ATGATATCCCACTTTTCACACCGCTCCAGTAGC-3' and 5'-ATAGATCTGGATTCATCCAACCCAGATGCAGC-3'), pMIC2-specific (5'-GGTACA-ACTTCACGGTGATGGCCC-3' and 5'-CACGGCGTGGACACTGTGATTCCC-3'), and pULBP1-specific (5'-GCGGCCTGCGATACTCACTCTCTTTGC-3' and 5'-GGAAGCTGGTCACAATCCGGTCACTCTCCC-3'). PCR products were run on a 1% agarose gel. Quantitative PCR was performed using Absolute QPCR SYBR Green Mix (ABgene, Hamburg, Germany) on a ABI Prism 7700 (Applied Biosystems, Rotkreuz, Switzerland) following the manufacturers protocol. Results were analyzed after the comparative threshold cycle method.

Western Blot

PEDSV.15 and PAEC cells were lysed using Cell Lytic M (Sigma). Cell lysates were analyzed by Western blot using standard protocols, and normal rabbit IgG and affinity purified anti-pULBP1 Ab were employed as primary polyclonal Ab. An HRP-conjugated secondary goat anti-rabbit Ab (Biorad, Reinach, Switzerland) was added and specific bands were visualized using Opti-4CN (Biorad).

ELISA and cellular ELISA

Maxisorp ELISA plates (Becton Dickinson) were coated 16 h before the assay using 1 μg of pULBP1-Fc or NKG2D protein in a volume of 100μl. Rabbit anti-pULBP1 polyclonal Ab was used as primary Ab with AP-conjugated goat anti rabbit IgG Ab (Sigma) as secondary Ab. For cellular ELISA, $5x10^3$ PEDSV.15 or $1x10^5$ NK cells were seeded 16 h before the assay in 96-well plates. NKG2D-Fc (R&D Systems), pULBP1-Fc, or pMIC2-Fc proteins were added (0.5μg per well) and incubated at 37°C for two h. An AP-conjugated goat anti-human IgG Ab (Sigma) was used as

secondary Ab. Then, 4-nitrophenyl phosphate (Merck, Dietikon, Switzerland) was added and the absorbance was measured at 405 nm.

5.2.4. Results

Human NKG2D binds to porcine endothelial cells and pULBP1

In order to test the ability of the activating human NK receptor NKG2D to directly interact with a ligand on pEC, the pEC line PEDSV.15 was incubated with the chimeric fusion protein NKG2D-Fc. NKG2D-Fc bound to PEDSV.15 cells in a dose-dependent manner as shown by cellular ELISA (Fig. 1A). Binding of NKG2D-Fc was also observed using primary porcine aortic endothelial cells (PAEC) (data not shown). Accordingly, FACS analysis revealed staining of PEDSV.15 cells with NKG2D-Fc, but not with an irrelevant Fc fusion protein (Fig. 1B). Preincubation of NKG2D-Fc with anti-NKG2D mAb abolished its binding to PEDSV.15 underscoring the specificity of the staining.

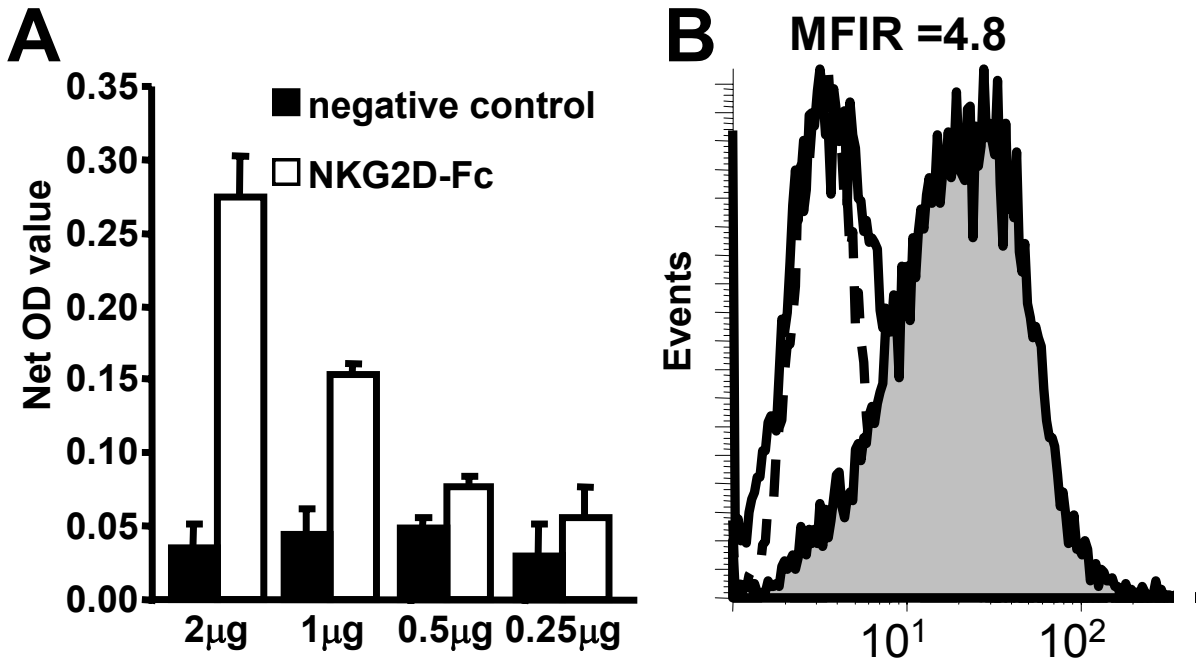

Figure 1: Human NKG2D-Fc binds to the surface of porcine endothelial cells. (A) Dose-dependent binding of human NKG2D-Fc to PEDSV.15 cells in cellular ELISA. Results are representative for one out of three independent experiments. Error bars represent standard deviations. (B) Binding of human NKG2D-Fc to PEDSV.15 cells as shown by flow cytometry (filled histogram), an irrelevant Fc fusion protein was used as negative control (empty histogram), binding of human NKG2D-Fc to PEDSV.15 cells was completely abrogated by preincubation with anti-NKG2D mAb (empty histogram, dashed line). MFIR were calculated by dividing the mean fluorescence intensity of NKG2D-Fc binding with the irrelevant Fc fusion protein binding.

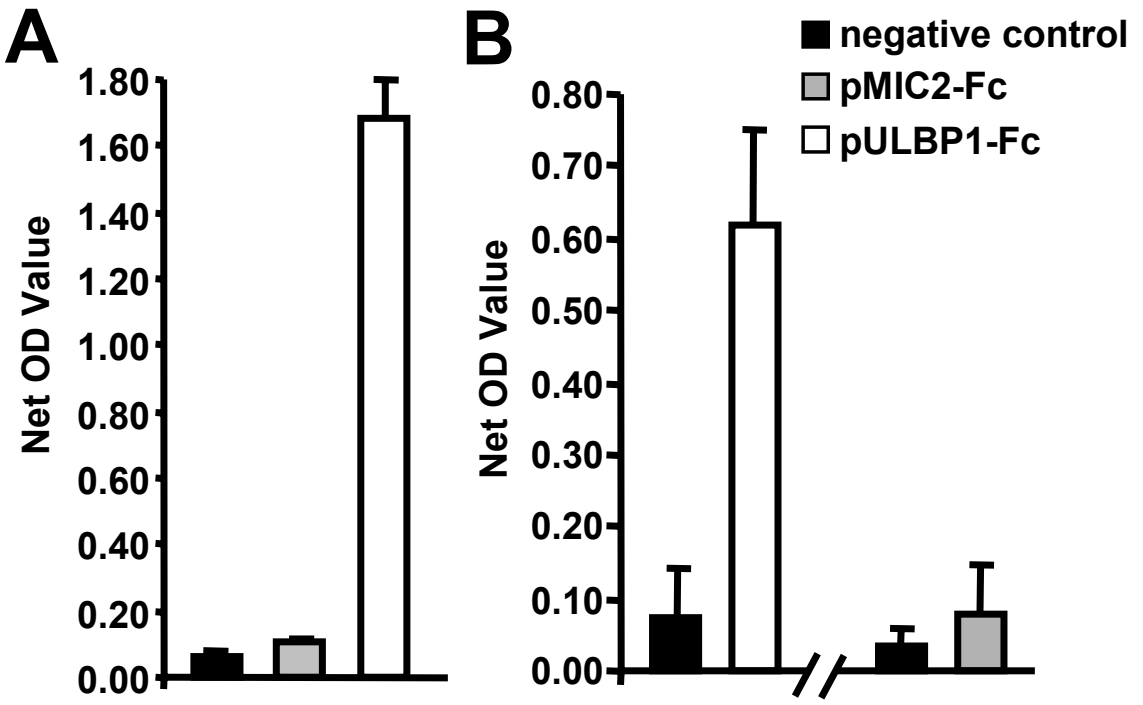

Figure 2: PULBP1-Fc but not pMIC2-Fc binds to recombinant human NKG2D and to the surface of human NK cells. (A) Binding of an irrelevant Fc fusion protein (black bar), pMIC2-Fc (grey bar), and pULBP1-Fc (white bar) to recombinant NKG2D protein measured by ELISA. Results are representative for one out of two independent experiments. Error bars represent standard deviations. (B) Binding of an irrelevant Fc fusion protein (black bar), pMIC2-Fc (grey bar), and pULBP1-Fc (white bar) to primary human NK cells measured by cellular ELISA. Results are representative for one out of two independent experiments. Error bars represent standard deviations.

Decreased susceptibility of PEDSV.15 to lysis by human NK cells following down-regulation of pULBP1 but not pMIC2 mRNA

To test whether pULBP1 and/or pMIC2 interact functionally with NKG2D on human NK cells, cytotoxicity assays were performed following transient down-regulation of the respective mRNAs using short interfering RNA (siRNA). PEDSV.15 cells were transfected with siRNA oligonucleotide duplexes specifically targeting pULBP1 and pMIC2 and a non-targeting control siRNA. Compared to PEDSV.15 cells transfected with control siRNA, a reduction of 39±23% (mean ± SD) for pULBP1 and of 58±20% for pMIC2 mRNA was observed 48 h post transfection by quantitative RT-PCR (data not shown). A reduced susceptibility of PEDSV.15 cells to NK92-mediated cytotoxicity was observed in ^{51}Cr-release cytotoxicity assays using pULBP1 siRNA, whereas pMIC2 siRNA transfection slightly increased the susceptibility (data not shown). Since mRNA levels were only transiently downregulated by siRNA, experiments were performed within 48 to 72 h post transfection. There were many variable parameters in these siRNA assays such as the unknown half-life of NKG2D ligands on the surface of pEC and the exact duration and extent of mRNA down-

regulation. Consequently, the optimal time point for functional assays was difficult to estimate.

Therefore, PEDSV.15 lines with stable down-regulation of mRNA were generated using short hairpin RNA (shRNA) expressing vectors. The reduction of pULBP1 mRNA was 46±11% (mean ± SD) in the PEDshU2 line, and 87±17% for pMIC2 mRNA in the PEDshM3 line, respectively, as demonstrated by quantitative RT-PCR (Fig. 3 A and B). NK cytotoxicity mediated by IL-2 activated human NK cells against PEDshU2 was reduced to a relative level of 70±3% (mean ± SEM; n=8) as compared to PEDsh control targets (Fig. 3C). Blocking with NKG2D mAb reduced NK cytotoxicity to a level of 40±3% regardless of whether PEDsh control or PEDshU2 were used as target cells (Fig. 3C). In contrast, PEDshM3 target cells were more susceptible to NK cytotoxicity (119±3%; n=9), and blocking with NKG2D mAb reduced the lysis by 48±3% and 52±2%, respectively, in comparison to the respective isotype control (Fig. 3D). These observations were consistent with the results obtained by transient siRNA transfection. The reduced NK susceptibility of PEDshU2 was pULBP1-specific and corresponded to the level of remaining pULBP1 expression. The increased NK susceptibility of PEDshM3 was independent of NKG2D. Taken together, these results show that pULBP1, but not pMIC2, appears to act as a functional ligand for human NKG2D.

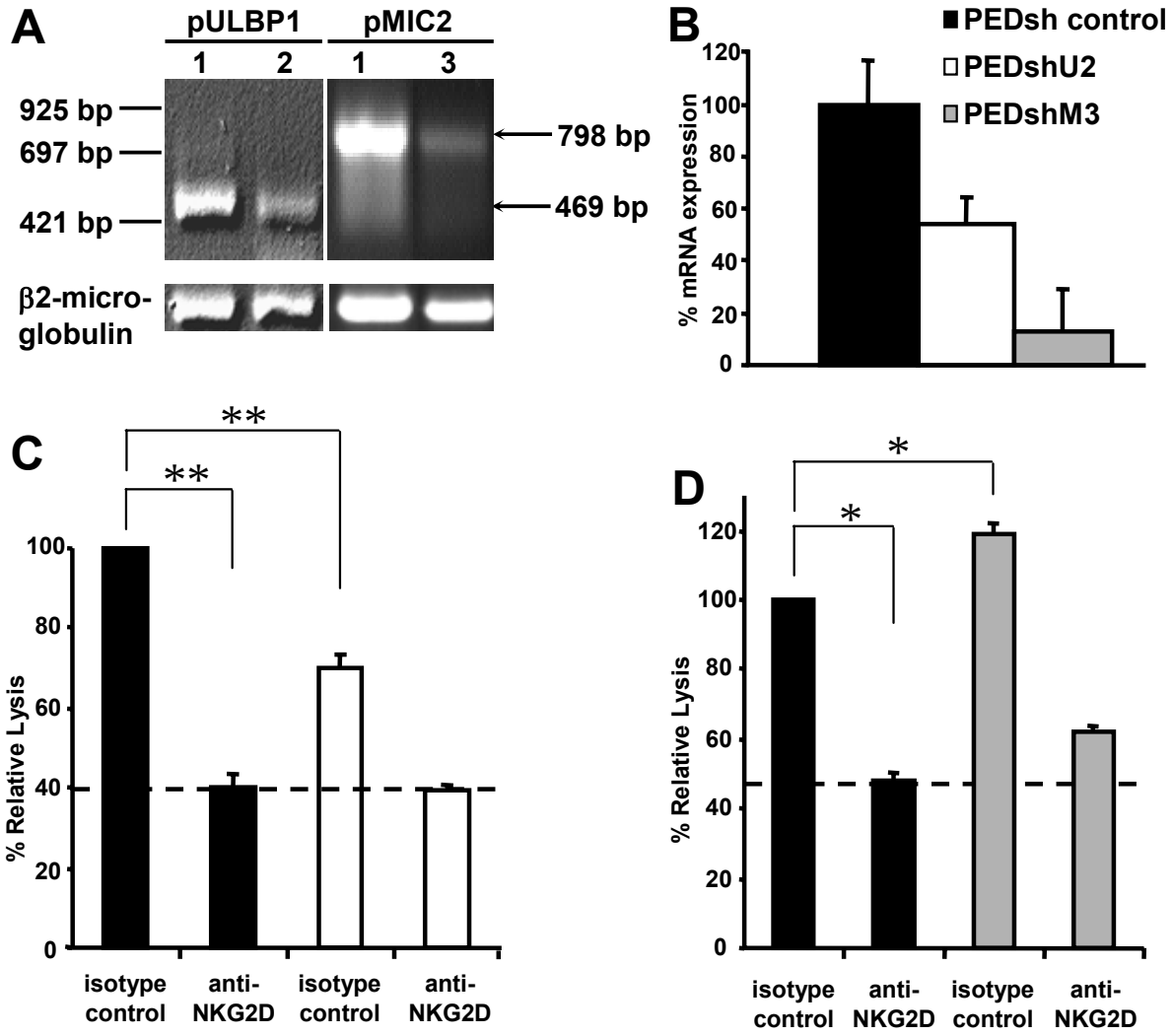

Figure 3: Decreased susceptibility of PEDSV.15 to human NK cytotoxicity following down-regulation of pULBP1 but not pMIC2 mRNA. (A) Agarose gel of RT-PCR products using RNA isolated from PEDsh control (lane 1), PEDshU2 (lane 2), or PEDshM3 (lane 3) as templates and either pULBP1- or pMIC2-specific primers (indicated above the lanes). Specific PCR products of 469 bp and 798 bp for pULBP1 and pMIC2 are depicted. β2-microglobulin-specific RT-PCR was used as positive and loading control. (B) Quantitative PCR analyzed with the comparative threshold analysis. Results reflect percentage of indicated mRNA level in comparison to PEDsh control. Error bars represent standard deviations. (C and D) Xenogeneic human NK cytotoxicity against PEDsh control (black bars), PEDshU2 (white bars), and PEDshM3 (grey bars) targets analyzed by 4 h ^{51}Cr-release cytotoxicity assays. The mouse IgG1 anti-NKG2D mAb 149810 was used for blocking. The percentage of relative lysis of PEDshU2 and PEDshM3 target cells in the presence of the indicated mAb was obtained by comparison to the lysis of PEDsh control targets in the presence of isotype control mAb (index=100). A summary of three independent experiments with eight different donors is shown. The mean relative cytotoxicity was calculated at four different E:T ratios (40:1 to 5:1), error bars represent SEM. Asterisks mark statistical significance in a student's t-test (*p<0.05, **p<0.01).

Generation of a polyclonal anti-pULBP1 antibody

In an effort to confirm pULBP1 surface expression on pEC by flow cytometry and to completely block activating signals elicited by pULBP1 in cytotoxicity assays, a polyclonal Ab against pULBP1 was generated. Following immunization of rabbits with

pULBP1-Fc a polyclonal pULBP1 Ab was purified from the serum of one rabbit. This Ab bound to pULBP1-Fc protein in ELISA whereas purified rabbit Ig obtained from preimmune serum did not (Fig. 4A). To further characterize the polyclonal Ab and to show pULBP1 specificity, PEDSV.15 and PAEC cell lysates were analyzed by Western blotting. As shown in Figure 4B a band of 55-60 kDa was detected by the affinity-purified polyclonal anti-pULBP1 Ab corresponding to the predicted size of pULBP1 dimers. In contrast, no band was seen with the control rabbit Ig obtained from preimmune serum (data not shown). Cell surface expression of pULBP1 on PEDSV.15 and PAEC cells was demonstrated by flow cytometry using the anti-pULBP1 polyclonal Ab (Fig. 4C). Providing additional support for the specificity of the Ab, HEK293 cells stably transfected with full-length pULBP1 were positively stained by anti-pULBP1 polyclonal Ab whereas untransfected HEK293 were negative (Fig. 4D). Finally, pULBP1 cell surface expression on the PEDshU2 obtained by shRNA transfection (described above) was reduced by 50% as compared to PEDsh control cells (data not shown), supporting the results obtained by quantitative RT-PCR (Fig. 2B). These data show that rabbit anti-pULBP1 polyclonal Ab specifically binds to pULBP1 expressed on the surface of porcine cells.

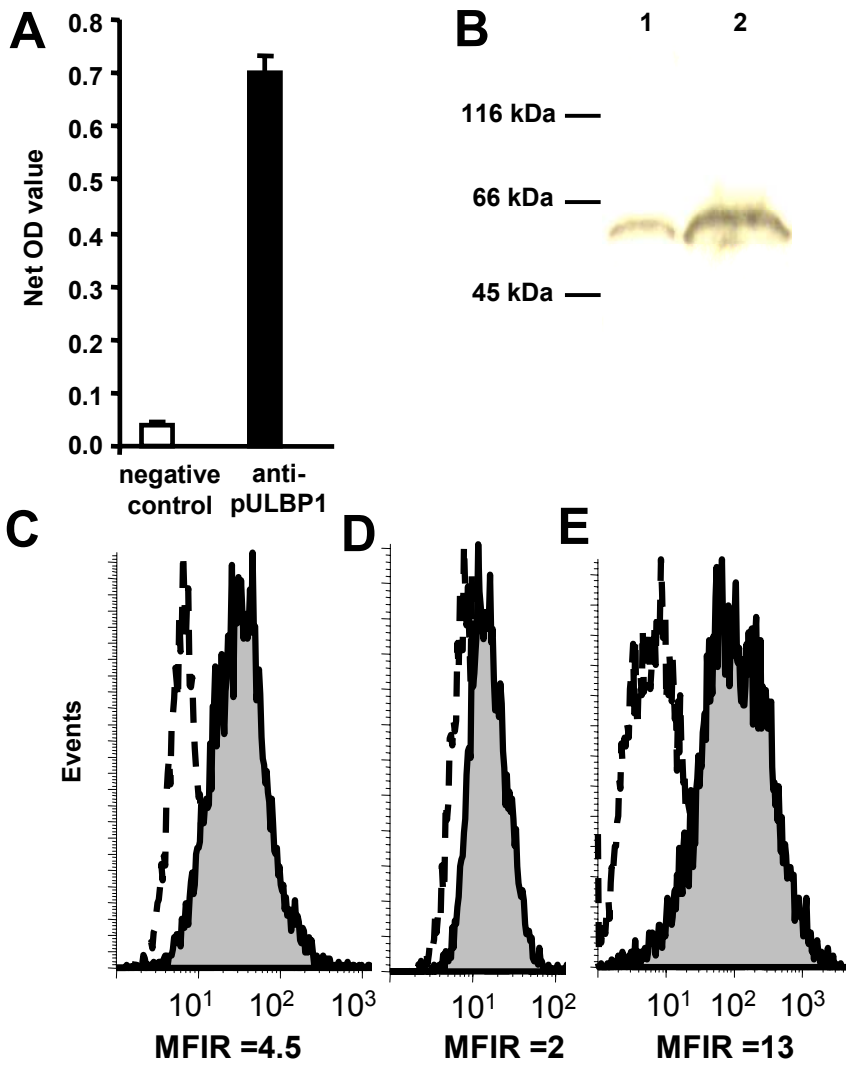

Figure 4: Characterization of polyclonal rabbit anti-pULBP1 Ab. (A) Binding of anti-pULBP1 Ab (black bar) to pULBP1-Fc measured by ELISA, normal rabbit IgG (white bar) was used as negative control. Net OD values are given and error bars represent standard deviations. (B) Western blotting of both PEDSV.15 (lane 1) and PAEC (lane 2) lysates using affinity-purified anti-pULBP1 Ab revealed a single band of 55-60 kDa. Results are representative for one out of two independent experiments. Binding of anti-pULBP1 polyclonal Ab (filled histograms) to (C) PEDSV.15 and (D) PAEC demonstrated by indirect flow cytometry. Staining with normal rabbit IgG was used as negative control (empty histograms, dashed line). (E) Binding of anti-pULBP1 polyclonal Ab to HEK 293 cells transfected with pULBP1 (filled histogram) and mock-transfected HEK293 cells (empty histogram, dashed line) shown by indirect flow cytometry.

Anti-pULBP1 polyclonal Ab completely blocks human NK cytotoxicity against porcine endothelial cells mediated through NKG2D

The ability of rabbit anti-pULBP1 polyclonal Ab to block functional pULBP1/NKG2D interactions was examined in cytotoxicity assays. Incubation of PEDSV.15 cells with anti-pULBP1 polyclonal Ab reduced NK cytotoxicity mediated by freshly isolated and IL-2 activated human NK cells by 67±13% (specific lysis±SEM) and 41±4%, respectively Fig. 5A,B). This level of inhibition correlated well with the blocking of NK

cytotoxicity observed with anti-NKG2D mAb preincubation of freshly isolated and IL-2 activated NK cells; 67±14% and 39±5% inhibition, respectively. Finally, as compared to single Ab blocking, combined Ab blocking of pULBP1 and NKG2D on porcine PEDSV.15 and human NK cells did not further enhance the inhibition of NK cytotoxicity. Similar results were obtained in cytotoxicity assays using primary porcine target cells (Fig.5C,D). Together, these findings not only demonstrate specific functional interactions between pULBP1 and human NKG2D, but also strongly suggest that pULBP1 is the predominant, if not the only, functional ligand for human NKG2D on porcine cells.

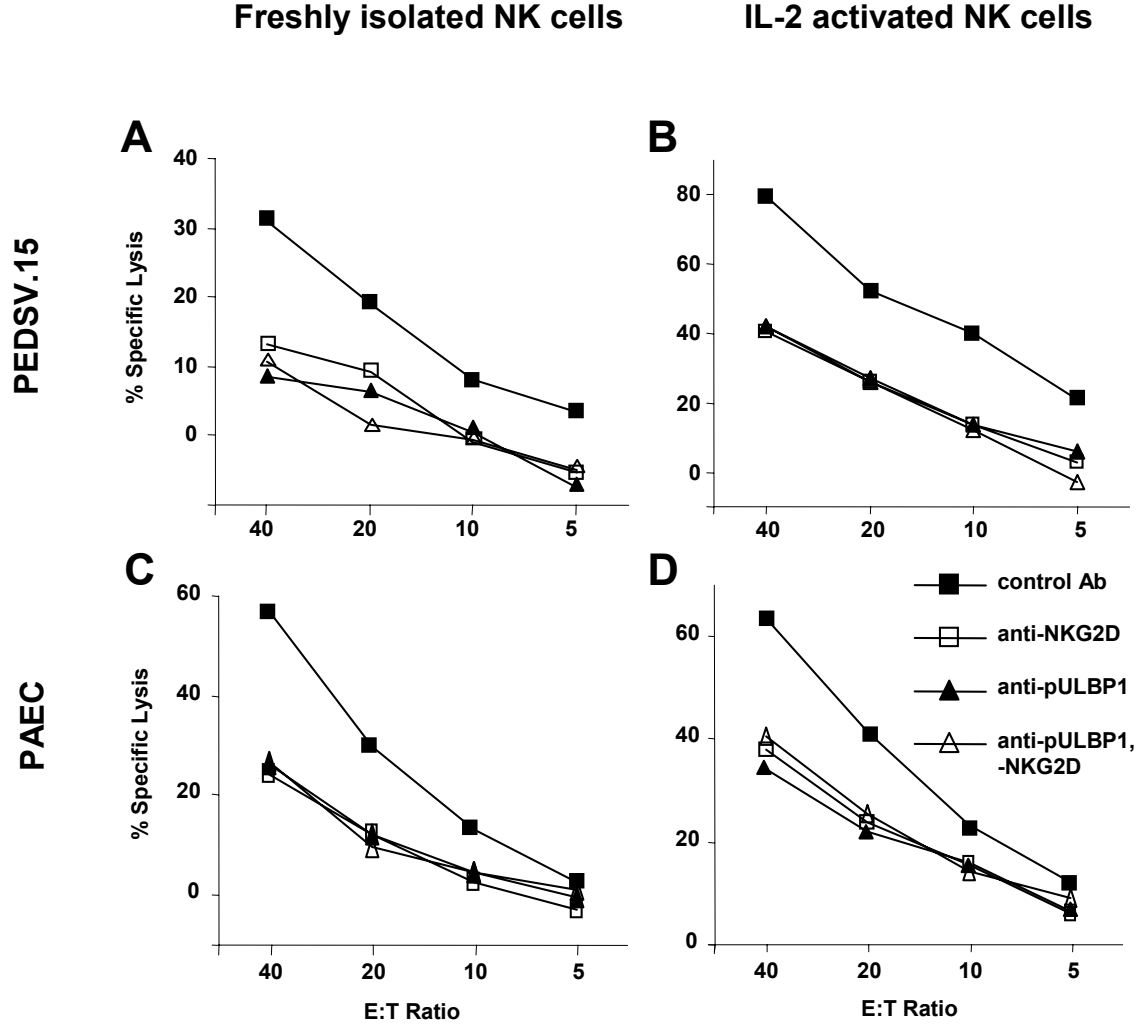

Figure 5: Anti-pULBP1 polyclonal Ab completely blocks human NK cytotoxicity against porcine endothelial cells mediated through NKG2D. NK cytotoxicity mediated either by freshly isolated (A and C) or IL-2 activated (B and D) human NK cells against PEDSV.15 (A and B) and PAEC (C and D) was tested in 4 h ^{51}Cr-release cytotoxicity assays. For blocking, anti-NKG2D (open squares), anti-pULBP1 (filled triangles), a combination of both (open triangles), and control Abs (filled squares) were used. To prevent ADCC the Fc receptor CD16 was blocked by incubation of NK cells with anti-CD16 mAb in all assays. Data shown represent one out of two independent experiments using four different NK cell donors (A and C), one out of six independent experiments using five different donors (B), and one out of three independent experiments with six different donors (D).

5.2.5. Discussion

NK cell-mediated rejection mechanisms may represent one of the remaining obstacles preventing the clinical application of pig-to-human xenotransplantation. As shown in a series of previous studies by several investigators, human NK cells are able to adhere to and activate pEC (35, 39, 48, 51). These interactions lead to morphological changes, the release of pro-inflammatory cytokines and endothelial damage. Whether these *in vitro* phenomena correspond to tissue damage and consequently to the loss of xenografts *in vivo* is still a matter of debate (54). Human NK cytotoxicity against pEC might depend on incompatible cross-species interactions between porcine MHC class I molecules and inhibitory human NK receptors (72). This notion was supported by the expression of HLA class I molecules on pEC and their complete protection from NK clones expressing the respective HLA-specific inhibitory NK receptor. However, only partial protection from human NK cytotoxicity mediated by polyclonal NK populations was observed (77, 110, 184, 185). Beside the lack of MHC class I inhibition, the recognition of putative porcine ligands by human activating NK receptors plays an important role in xenogeneic NK cytotoxicity. Recently, we demonstrated that lysis of pEC mediated by freshly isolated human NK cells is mainly triggered by NKG2D (81). In contrast, lysis mediated by IL-2 activated human NK cells depends on both NKG2D and NKp44. These results predicted the presence of one or several porcine ligand(s) for human NKG2D expressed on pEC.

Here we analyzed two recently identified porcine homologues of human NKG2D ligands and demonstrate that pULBP1, but not pMIC2, functionally interacts with human NKG2D. Using an algorithm to score potential ligands (153) pULBP1 was predicted to bind human NKG2D using both the crystal structure of human NKG2D/human ULBP3 and human NKG2D/mouse Rae-1β interactions as template, whereas pMIC2 was predicted to bind NKG2D only using the structure of human NKG2D/human ULBP3 as template (data not shown). Intriguingly, a previous study showed no binding of pULBP1-Fc to the human NK cell line NKL by flow cytometry whereas binding to porcine PBMC was revealed (155). It was concluded then that pULBP1 does not interact with human NKG2D. The discrepant data regarding pULBP1 binding to human NKG2D might be explained by different experimental conditions. First, the relatively low affinity of Fc fusion proteins used in the previous report may prevent positive staining in flow cytometry assays. In the present paper

pULBP1 binding to human NK cells was demonstrated by cellular ELISA. Second, primary human NK cells as well as the NK92 cell line were used, which may exhibit different surface molecule expression patterns as compared to the NKL cell line. Finally, the inability of the soluble Fc fusion proteins to bind to human NK cells may be due to aberrant folding as compared to the membrane-bound form on the cell surface.

However, these binding results did not shed light on the functionality of human NKG2D/pULBP1 interactions which we further explored by downregulation of mRNA by stable transfection of shRNA expressing vectors (186). These experiments added further evidence that pULBP1 acts as a ligand for human NKG2D. The fact that not a complete abrogation of the NKG2D-mediated cytotoxicity was observed was probably due to the remaining pULBP1 mRNA (Fig. 3B) and surface protein (data not shown). On the other hand, the reason why downregulation of pMIC2 rendered PEDSV.15 cells more susceptible to NK-mediated killing remains unclear. Since pMIC2 is closely related to MHC class I, we tested whether a protecting signal was delivered by pMIC2 through one of the MHC-specific inhibitory NK receptors on human NK cells. In such a situation, downregulation of pMIC2 would interfere with these inhibiting signals leading to an enhanced NK cytotoxicity. However, blocking receptor-ligand interaction using specific mAb against several MHC-specific inhibitory NK receptors (KIR2DL2, KIR2DL3, KIR3DL1, ILT2) did not provide proof for this hypothesis (data not shown). Another possible explanation for the observed increase in lysis of pEC following pMIC2 downregulation are potential RNAi off-target effects that were reported by Jackson et al (187). Further studies are necessary to understand how pMIC2 down-regulation, independent of NKG2D signaling, increases susceptibility to lysis by human NK cells.

Complete inhibition of xenogeneic NK cytotoxicity triggered by pULBP1 was achieved by polyclonal anti-pULBP1 Ab blocking. The relative inhibition was stronger when freshly isolated NK cells were used as compared to IL-2 activated NK cells. This finding was expected, since only the latter express NKp44 which is also involved in xenogeneic NK cytotoxicity (81, 143). However, the incomplete blocking of xenogeneic NK cytotoxicity observed using freshly isolated NK effectors indicated the presence of yet unidentified interactions between porcine ligands with human

activating NK receptors. The identification of these ligand/receptor pairs as well as the porcine ligands for NKp44 is currently being addressed in order to completely overcome NK-mediated xenograft rejection.

This study suggests that pULBP1 is the predominant, if not the only, functional ligand for human NKG2D on porcine cells. Porcine EC express at least one other ULBP-like transcript (pULBP2) although at the mRNA level its expression is about 20-fold less then pULBP1. Also, preliminary analysis of a porcine bacteria artificial chromosome clone suggests several additional loci encoding ULBP-like proteins (own unpublished observations). In this regard pigs appear similar to humans where several ULBP and MIC proteins serve as ligands for NKG2D (150, 151), and also mice, cattle and primates which express several NKG2D ligands (188, 189). The redundancy of the NKG2D system within a species might be driven by immune evasion mechanisms of pathogens such as CMV. However, considering that no evolutionary pressure acted on interactions between human and porcine molecules, the lack of redundancy across the species barrier (i.e. pULBP1 being the only ligand for human NKG2D) is not very surprising and sheds more light on the molecular incompatibilities between man and pig already identified. On the other hand, the molecular compatibility between human NKG2D and pULBP1 might help to prevent zoonoses and infections of porcine xenografts by human pathogens. Therefore, future studies need to address the important question of whether the elimination of pULBP1 might pose a risk by interfering with anti-infectious NK cell responses.

In conclusion, similar to hyperacute xenograft rejection which has been largely overcome using organs derived from alphaGal knockout pigs, the elimination of pULBP1 on porcine tissues might have important implications as a complementary approach to protect porcine xenografts from human NK cell responses. Here we focused on direct human NK cytotoxicity against pEC, but there are other potentially harmful interactions between human NK cells and pEC. These include direct cellular activation of pEC, the release of chemokines and cytokines such as porcine TNF-α and IL-8 leading to further EC activation and the recruitment of additional human leukocytes to the graft. Moreover, the proposed protection of pEC from human NK cytotoxicity by interfering with pULBP1/human NKG2D interactions indicates a possible use to facilitate hematopoietic stem cell xenotransplantation, which has

been proposed as a method to induce xenogeneic tolerance (190, 191). Finally, the potential of pULBP1 to induce additional xenogeneic human NK cell responses such as IFN-γ secretion by triggering NKG2D remains to be considered.

5.2.6. Acknowledgements

We would like to thank I. Cummings (University Hospital Zürich, Switzerland) for technical assistance and all healthy volunteers for blood donations. J.J. Eloranta (University Hospital Zürich, Switzerland) is recognized for helpful discussions and technical assistance. M.K.J. Schneider (University Hospital Zürich, Switzerland) and W. Held (University of Lausanne) are acknowledged for careful reading of the manuscript and helpful comments.

5.3. Characterization of porcine UL-16 binding protein 1 (pULBP1) endothelial cell surface expression using a novel anti-pULBP1 monoclonal antibody

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Author contributions: BGL, AS, and LLI designed, performed, and analyzed the experiments. BGL wrote the manuscript. MKJS and NM contributed to CMV work and paper writing. JDS supervised the project and contributed to paper writing.

manuscript in preparation

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Key words: NK cells, pULBP1, Xenotransplantation

Abbreviations: αGal: galactose-α-1,3-galactose; ADCC: Ab-dependent cell-mediated cytotoxicity; CMV: cytomegalovirus; NCR: natural cytotoxicity receptors; KIR: killer immunoglobulin-like receptors; MFIR: geometric mean fluorescence intensity ratio; MIC: MHC class I chain-related protein; pEC: porcine endothelial cells; PFT: porcine fallopian tube; pULBP1: porcine UL-16 binding protein 1; SLA: swine leukocyte antigen

5.3.1. Abstract

NK cells are tightly regulated through signals mediated by inhibiting and activating receptors expressed on their cell surface. *In vitro*, freshly isolated human NK cells kill porcine endothelial cells (pEC) through the interaction of the activating receptor NKG2D and its corresponding ligand on pEC, porcine UL-16 binding protein 1 (pULBP1). The aims of the study were (I) to generate a mAb against pULBP1, and (II) to characterize pULBP1 cell-surface expression on primary porcine aortic endothelial cells (PAEC) after treatment with various stimuli. In order to obtain mAb against pEC surface molecules, C57BL/6 mice were immunized with the pEC line PEDSV.15. Porcine ULBP1 cell surface expression on primary PAEC was measured with cellular ELISA. Primary PAEC were either left untreated or stimulated with human or porcine cytokines (IFN-γ, TNF-α), co-cultured with human serum, cultured under hypoxic conditions, or infected with human or porcine cytomegalovirus (CMV). The mouse anti-pULBP1 mAb clone aE5-63 specifically stained pULBP1 on the pEC surface. IFN-γstimulation did not change pULBP1 cell surface expression, whereas human and porcine TNF-αstimulation as well as human and porcine CMV infection significantly decreased pULBP1 cell surface expression on primary PAEC. Coculture of primary PAEC for 24h with human serum strongly increased the pULBP1 cell surface expression in a dose-dependent manner. Also, culture under hypoxic conditions led to a slight but significant increase of pULBP1 cell surface expression. In conclusion, (I) the novel anti-pULBP1 mAb aE5-63 is specific and may represent a useful tool for the study of NKG2D-mediated responses in xenotransplantation, and (II) pULBP1 cell surface expression on primary PAEC is tightly regulated in response to various stimuli.

5.3.2. Introduction

NK cells participate in the immune response against solid organ allo- and xenografts (47, 181) and are tightly regulated through signals mediated by inhibiting and activating receptors expressed on their cell-surface (192). Many of the inhibitory NK receptors recognize MHC class I molecules and therefore allow NK cells to discriminate between normal self, non-self and altered cells (116). Porcine endothelial cells (pEC) are susceptible to human NK cell-mediated lysis possibly due to the inability of swine leukocyte antigen (SLA) class I molecules to signal through human NK inhibitory receptors (72, 74). On the other hand, the activation of human

NK cells by potential ligands expressed on pEC could be even more important. Activating receptors on NK cells include NKp30, NKp44, and NKp46 (119), collectively named natural cytotoxicity receptors (NCR), and the C-type lectin receptor NKG2D (120). While NKp30 and NKp46 are detected on all NK cells regardless of their activation status, NKp44 is selectively expressed by activated NK cells (143). As recently reported, human NK cytotoxicity against pEC is mediated primarily through NKG2D and NKp44, whereas NKp30 and NKp46 are not involved (81).

NKG2D is constitutively expressed on NK, NKT, and $CD8^+$ T cells as a homodimeric receptor associated with the transmembrane adaptor molecule DAP10 (148). Over the past few years several regulatory mechanisms of NKG2D surface expression have been reported (120, 182). Cellular ligands of NKG2D include the stress-inducible MHC class I chain-related proteins A/B (MICA/B) (151) and UL16-binding proteins (ULBP) (150). The latter were identified based on their ability to bind the human CMV (hCMV) glycoprotein UL16. Although these GPI-linked proteins are distantly related to members of the HLA class I family possessing α1 and α2, but not α3 domains (152), they are unable to present peptides (153). ULBP1 and -2 cell surface expression is decreased following hCMV infection due to the association with the CMV protein UL16 in the endoplasmic reticulum or *cis*-Golgi compartments (193, 194). Therefore, maturation and transport of the proteins to the cell surface is prevented. Both MICA and MICB are transmembrane proteins and possess all three α-domains. Similar to ULBP, MICA and MICB do not bind β2-microglobulin or present peptides (154).

Only recently, porcine ULBP1 (pULBP1) was cloned and characterized as a homologue of human ULBP (155). Furthermore, pULBP1 was shown to be the predominant, if not only, ligand for the human NK activating receptor NKG2D (157). Phylogenetic analysis places pULBP1 evolutionarily close to the bovine ULBP-like genes MHCLA1 and MHCLA2. It exhibits 35-52% amino acid identity to human ULBP including a relatively high level of conservation at positions predicted to make contact with human NKG2D (153). Southern blot analysis suggested that only one pULBP exists in the pig genome, which is in sharp contrast to the much higher number of

ULBP genes, at least six, that were described in humans. Transient and stable downregulation of pULBP1 mRNA in pEC using short interfering RNA resulted in a partial inhibition of xenogeneic NK cytotoxicity through NKG2D in cytotoxicity assays. Furthermore, human NK cytotoxicity against pEC mediated by freshly isolated or IL-2-activated NK cells through NKG2D was completely blocked using anti-pULBP1 polyclonal Ab (157).

The aim of this study was to generate a specific anti-pULBP1 mAb. Furthermore, the influence of various stimuli, including cytokines and human serum, and transplantation related phenomenons, such as hypoxia/reoxygenation and CMV infection, on pULBP1 cell surface expression was analyzed.

5.3.3. Materials and Methods

Cells

Primary PAEC and primary PAEC α-1,3-galactosyltransferase (αGAL) knockout (KO) were isolated from a normal and an αGAL KO pig, respectively, following standard procedures (166) and cultured as described earlier (44). The SV40-immortalized aortic pEC line PEDSV.15 was established and characterized in our laboratory (163). HEK293/pULBP1 cells were generated as described earlier (157). Hybridomas were cultured in IMDM (Invitrogen AG, Basel, Switzerland) supplemented with 10% FCS (PAA Laboratories, Luzern, Switzerland), 1% penicillin/streptomycin (Invitrogen), and 50mM β-mercaptoethanol (Sigma, Buchs, Switzerland).

Flow cytometry

Surface expression of pULBP1 on PAEC, PEDSV.15 and HEK293/pULBP1 cells was analyzed on a FACScanto (Becton Dickinson, Basel, Switzerland) by indirect immunofluorescence using the primary mouse mAb aE5-63 (generated as described in the Results section) or the polyclonal rabbit anti-pULBP1 Ab (157). PE-conjugated mouse anti-mouse IgM^b Ab (Becton Dickinson, Basel, Switzerland) and FITC-conjugated goat anti-rabbit IgG Ab (Sigma) were used as secondary Ab. An irrelevant, isotype-matched control mAb (G155-228, mouse IgG1, BD Pharmingen) was used as control for aE5-63 mAb stainings, and normal rabbit IgG (R&D Systems)

was used as control for the polyclonal rabbit Ab. Cells were fixed with 2% PFA (Sigma) prior to mAb incubation. To compare the levels of surface expression, the geometric mean fluorescence intensity (MFI) ratios (MFIR) were calculated by dividing the MFI of staining with the mAb of interest with the MFI of the control mAb.

Monoclonal Ab production

After initial immunization of C57BL/6 mice (H-2^b, obtained from the Institute of Laboratory Animal Science, University of Zürich, Zürich, Switzerland) with $5x10^6$ PEDSV.15 cells i.v. and 10^7 PEDSV.15 cells i.p. in PBS, mice received two booster immunizations (10^7 PEDSV.15 cells i.p.) at 2-week intervals. Final immunization was performed 1 month after the last booster immunization on day minus 4 (four days prior to removal of the spleen) by another 10^7 PEDSV.15 i.p. Peritoneal macrophages from three Balb/c mice (H-2^d, obtained from Charles River Breeding Laboratories, Hannover, Germany) were harvested on day minus 1 and plated on six 96-well flat bottom tissue culture plates to support the efficiency of the fusion by removing cell debris and stimulating cell proliferation by cytokine production. On day zero, fusion of splenocytes of immunized mice to the mouse myeloma cell line X63Ag8.653 was performed according to standard procedures (195). Hybridoma supernatants were screened for their ability to bind PEDSV.15 cells in a cellular ELISA and positive hybridomas were subcloned by limiting dilution cloning. Monoclonal hybridoma supernatants were screened for their ability to bind pULBP1-Fc fusion protein and inability to bind human IgG in ELISA. The hybridoma supernatant was purified using a Mannan Binding Protein column (Pierce, Lausanne, Switzerland) following the manufacturer's protocol.

ELISA and cellular ELISA

Maxisorp ELISA plates (Becton Dickinson) were coated 16 h before starting of the assay using 1 μg of pULBP1-Fc or human IgG in a volume of 100μl. The mouse anti-pULBP1 mAb aE5-63 was used as primary Ab with AP-conjugated goat anti-mouse polyvalent Ig, or HRP-conjugated mouse anti-mouse IgM^a, mouse anti-mouse IgM^b (Sigma), rat anti-mouse IgD (The binding site, Birmingham, UK), rat anti-mouse IgE (Southern Biotech, Reinach, Switzerland), goat anti-mouse IgA (Sigma), rabbit anti-mouse IgG1 (Zymed, Basel, Switzerland), goat anti-mouse IgG2a (Southern

Biotech), rabbit anti-mouse IgG2b (Zymed), goat anti-mouse IgG2c (Bethyl, Lausen, Switzerland) and rat anti-mouse IgG3 (The binding site) as secondary Abs. For cellular ELISA, $2.5x10^3$ primary PAEC cells were seeded 24 h before starting of the assay in 96-well plates. Cells forming intact monolayers were fixed with 2% PFA and incubated at 37°C for 2 h with isotype control or aE5-63 mAb. Goat anti-mouse polyvalent Ig Ab was used as secondary Ab. Finally, 4-nitrophenyl phosphate (Merck, Dietikon, Switzerland) was added and the absorbance was measured at 405 nm. Cells were either left untreated or were treated as follows: 48h with 1000U/ml human IFN-γ (Peprotech, London, UK), or 10ng/ml porcine IFN-γ (Innogenetics, Zwijndrecht, Belgium); 6 h with 1000U/ml human TNF-α or 100 ng/ml porcine TNF-α (both Peprotech); 6 h or 24 h co-culture with heat-inactivated human serum (56°C, 30 min) observed from healthy donors or with αGAL absorbed heat-inactivated human serum (179); 10 h incubation at hypoxic conditions (1% O_2, 5% CO_2, 94% N_2) and either no or subsequent re-oxygenation for 1 h, 2 h, 4 h; 8 h, or 48 h; or infection with human and porcine CMV for 4 hours.

Human and porcine CMV infection

For infection with human CMV, a laboratory-type strain Ad 169, (American Type Culture Collection, Rockville, MD, USA) was cultured in human embryonic fibroblasts. Porcine CMV was cultivated from a respiratory isolate from an immunosuppressed Massachusetts General Hospital miniature swine. The virus was grown on confluent porcine Fallopian tube (PFT) endothelial cells (a kind gift of J. Fishman, Massachusetts General Hospital, Harvard Medical School) at 37°C,, as described (196) Viral inocula were prepared by removing medium from infected cell cultures. The medium was centrifuged at 12,000 *g* for 2 min, the resulting supernatant was filtered through a 0.45 μm syringe filter (Fisher Scientific SA, Wohlen, Switzerland), and aliquots were stored at -70°C. Mock infections were made using medium from uninfected human embryonic fibroblasts and PFT cells, respectively, processed identically to the infected medium. Human CMV infection (MOI=1) was documented by the immunocytological staining of the pp65 protein, a matrix structural phosphoprotein of 65 kDa, which appears in the nuclei of infected cells within one hour post infection, and represents uptake from the viral innoculum.

Porcine CMV infection (MOI=170) was shown by increasing titers of porcine CMV by quantitative PCR,as described previously (197).

5.3.4. Results

Generation of a mouse monoclonal anti-pULBP1 Ab

Mice were immunized after the protocol described in materials and methods. Supernatants of growing hybridomas were tested for their ability to bind to PEDSV.15 cells in a cellular ELISA. Of the hybridoma supernatants, 21 bound to PEDSV.15 cells and were subcloned resulting in 328 monoclonal hybridomas, 104 of which bound to PEDSV.15 cells. Supernatant from one clone, designated as aE5-63, bound to pULBP1-Fc fusion protein in ELISA but showed no binding to human IgG (Fig 1A). The mAb aE5-63 was of the IgM^b isotype as revealed in an ELISA using secondary Ab against all different mouse immunoglobulin isotypes (Fig. 1B). Therefore, a mannan binding protein column was used to specifically purify this mAb. The purified mAb aE5-63 bound PEDSV.15 cells in a cellular ELISA (Fig. 1C). Providing additional support for the specificity of the mAb, PFA-fixed HEK293 cells stably transfected with full-length pULBP1 were positively stained by this mAb whereas mock-transfected HEK293 were negative (Fig. 1D). Furthermore, the mAb aE5-63 stained PFA-fixed primary PAEC (Fig. 1E), PEDSV.15 cells (Fig. 1F), the porcine bone marrow-derived aortic endothelial cell line 2A2, and the porcine lymphoblastoid cell line 13271.10 cells in FACS (data not shown). FACS stainings were only achieved with PFA-fixed but not unfixed cells. Western blots of pULBP1-Fc fusion protein revealed the same bands using either anti-human IgG Ab or the anti-pULBP1 mAb aE5-63 (data not shown). Altogether, these data prove that the mouse anti-pULBP1 mAb clone aE5-63 specifically stains pULBP1.

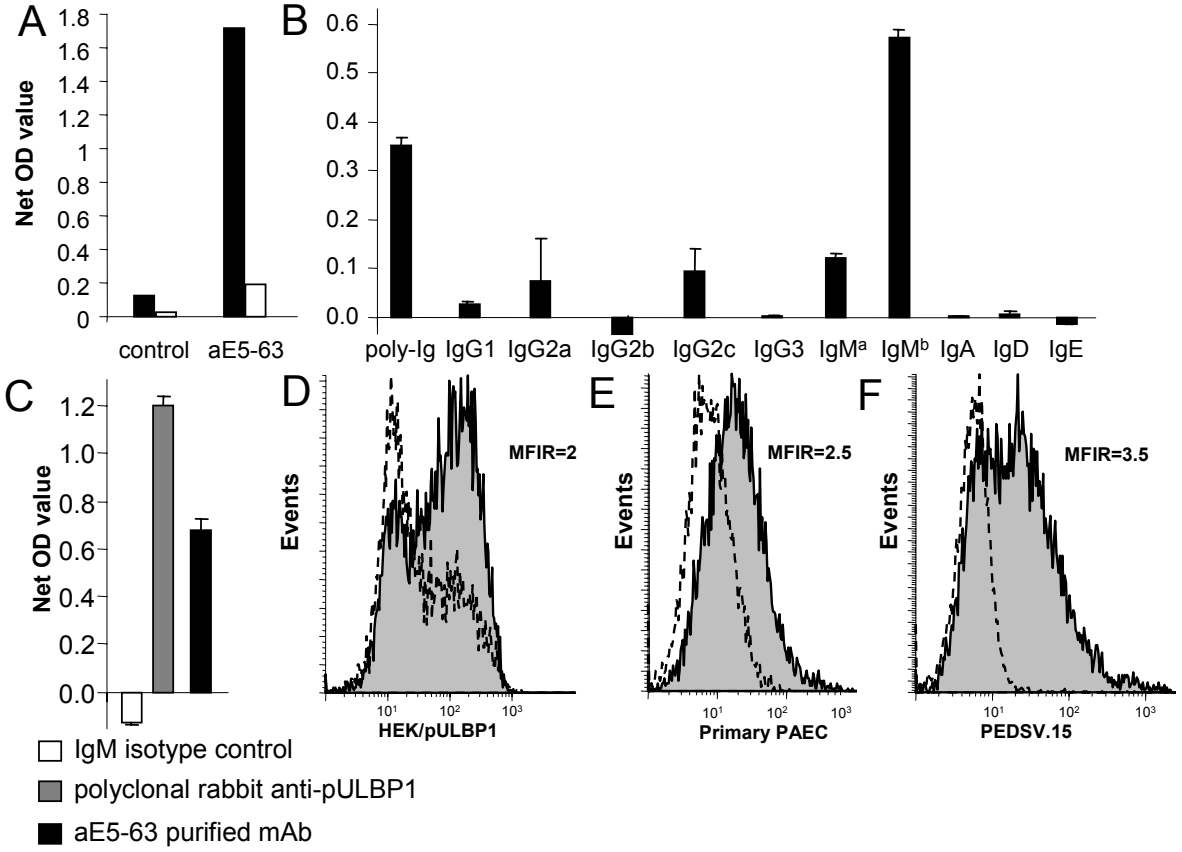

Figure 1: Characterization of the monoclonal anti-pULBP1 Ab aE5-63. (A) Binding of aE5-63 hybridoma supernatant to pULBP1-Fc but not to human IgG as measured by ELISA. ELISA plates were coated either with pULBP1-Fc (black bars) or human IgG (white bars). Hybridoma supernatant not binding PEDSV.15 cells was used as a negative control. (B) Designation of the isotype of aE5-63 hybridoma supernatant by ELISA. Error bars represent SEM. (C) Binding of purified anti-pULBP1 mAb aE5-63 (black bar) to PEDSV.15 cells in cellular ELISA. Isotype-matched Ab (white bar) and polyclonal anti-pULBP1 Ab (grey bar) were used as negative and positive controls, respectively. Error bars represent standard deviations. .(D-F) Binding of anti-pULBP1 mAb to HEK 293 cells transfected with pULBP1 (D), primary PAEC (E), and PEDSV.15 cells (F) shown by indirect flow cytometry (filled histograms). Empty dashed histograms depict binding of anti-pULBP1 mAb on mock-transfected HEK293 cells (D) or of isotype-matched control mAb on primary PAEC (E) and PEDSV.15 cells (F).

TNF-α stimulation decreases pULBP1 cell surface expression on primary PAEC

Porcine IFN-γ and human TNF-α induce the expression of SLA and adhesion molecules on pEC (198). To examine whether cytokine stimulation also influences pULBP1 cell surface expression, primary PAEC were stimulated with human or porcine IFN-γ, or human or porcine TNF-α. Surface expression of pULBP1 on primary PAEC was measured by cellular ELISA using the anti-pULBP1 mAb aE5-63. As expected, human IFN-γ stimulation did not show any effect, since porcine cells lack a receptor for human IFN-γ (198). However, porcine IFN-γ also did not influence pULBP1 cell surface expression. On the other hand, human as well as porcine TNF-α significantly decreased pULBP1 cell surface expression on primary PAEC (31±5%

and 33±3%, respectively; mean percentage decrease of pULBP1 cell surface expression as compared to untreated cells ±SEM, n=18) (Figure 2). Thus, TNF-α but not IFN-γ stimulation has an influence on pULBP1 cell surface expression on primary PAEC.

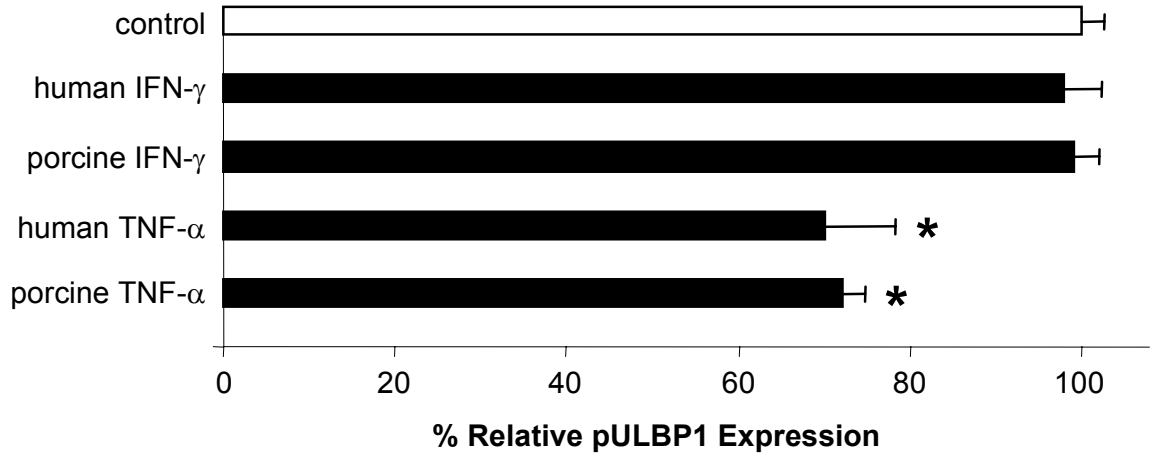

Figure 2: TNF-αstimulation decreases pULBP1 cell surface expression on primary PAEC. Shown is the relative percentage of pULBP1 cell surface expression after cytokine stimulation as compared to untreated cells (index=100). Error bars represent SEM. Asterisks depict statistical significance in a student's t-test (*p<0.0005, n=18).

Human serum increases pULBP1 cell surface expression on primary PAEC

In solid organ xenotransplantation, pEC represent the first line of contact between the graft and the immune system of the recipient. Therefore, we analyzed, whether the binding of human anti-pig xenoreactive Ab in the serum of a healthy donor has an influence on pULBP1 cell surface expression. Whereas the co-culture of primary PAEC with human serum for 6 h did not significantly change pULBP1 cell surface expression (data not shown), co-culture for 24 h led to a strong increase dependent on the amount of human serum added to the cells (Figure 3A). The mean increase of pULBP1 cell surface expression as compared to untreated cells was 49±7%, 75±19%, and 91±17% (±SEM, n=12) after coculture with 12.5%, 25%, and 50% human serum, respectively. No significant increase of pULBP1 cell surface expression was observed after coculture of primary PAEC with αGAL-absorbed human serum (Fig. 3B). Furthermore, co-culture of primary PAEC αGAL KO cells with normal as well as αGAL-absorbed human serum did not significantly change pULBP1 cell surface expression (data not shown). Therefore, the binding of

xenoreactive Ab in human serum to cell surface molecules on primary PAEC, the majority directed against αGAL, strongly increased pULBP1 cell surface expression.

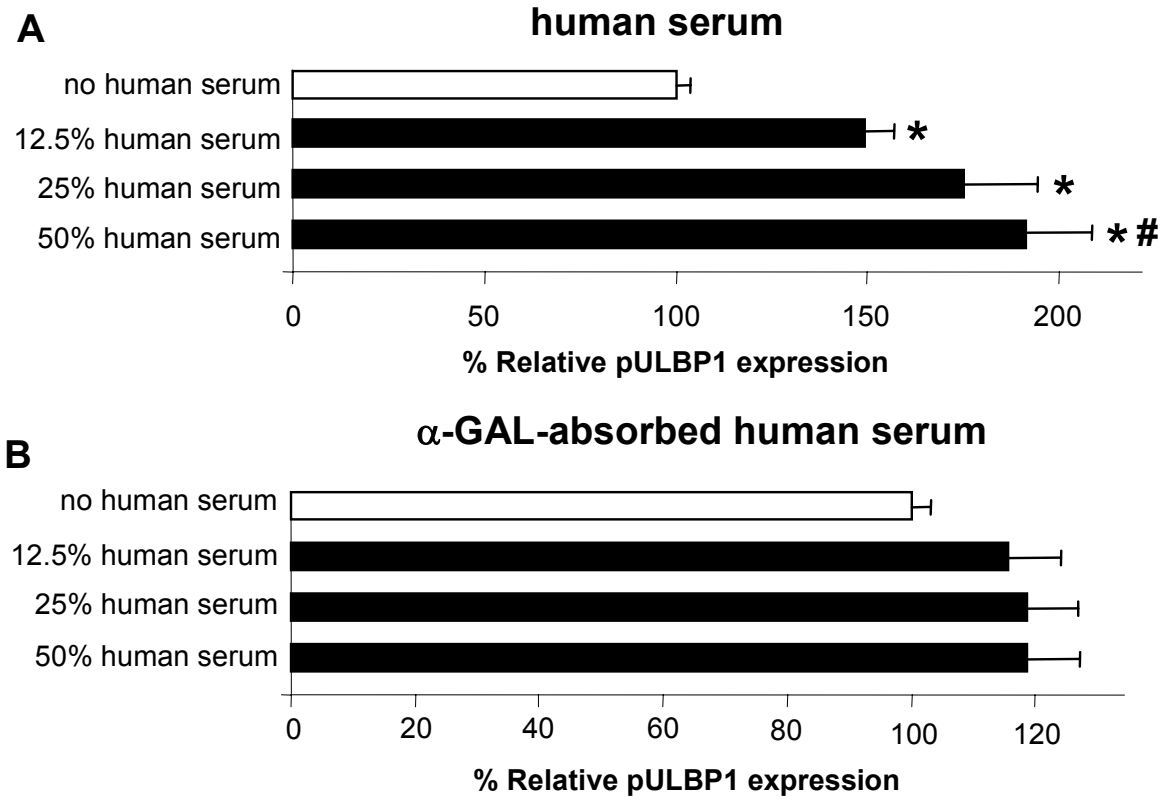

Figure 3: Human serum increases pULBP1 cell surface expression on primary PAEC. Shown is the relative percentage of pULBP1 cell surface expression after co-culture with human serum (A) and αGAL absorbed human serum (B) as compared to untreated cells (index=100). Error bars represent SEM. Asterisks and rhomb depict statistical significance in a student's t-test for human serum stimulation versus control, *$p<0.01$, n=12, and for 50% versus 12.5% human serum stimulation, #$p<0.05$, n=12, respectively.

Hypoxia/re-oxygenation increases pULBP1 cell surface expression on primary PAEC

Susceptibility of human microvascular EC to NK cytotoxicity was shown to be slighty reduced by hypoxia/reoxygenation in a model analyzing ischemia/reperfusion injury (199). Therefore, we tested whether pULBP1 cell surface expression on primary PAEC was influenced by hypoxia/reoxygenation. A slight but significant increase of approximately 20% of pULBP1 cell surface expression was observed after 8 h of hypoxia as well as after reoxygenation for 2 h, 4 h (Fig.4), and 8 h (data not shown). After 24 h of reoxygenation, pULBP1 cell surface expression returned to the basal level (Fig. 4). Thus, pULBP1 cell surface expression is slightly but significantly increased after of hypoxia/reoxygenation.

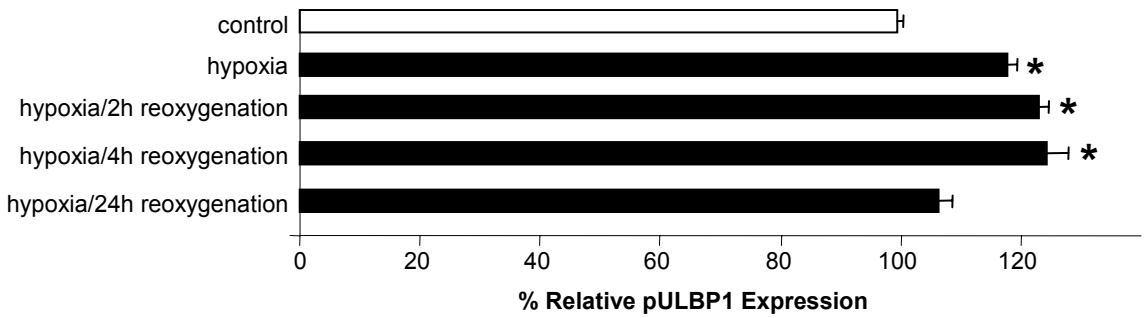

Figure 4: Hypoxia/reoxygenation increases pULBP1 cell surface expression on primary PAEC. Shown is the relative percentage of pULBP1 cell surface expression after hypoxia/re-oxygenation as compared to untreated cells (index=100). Error bars represent SEM. Asterisks depict statistical significance in a student's t-test (*p<0.005, n=12).

Human and porcine CMV infection decreases pULBP1 cell surface expression on primary PAEC

Human CMV infection is known to decrease the expression of the human NKG2D ligands ULBP1, -2, and MICB (194). Therefore, we tested whether pULBP1 on porcine cells is also decreased upon human or porcine CMV infection. Indeed, at 48 h and 72 h post infection with either human or porcine CMV, a slight but significant decrease of surface pULBP1 was observed (Fig. 5). No significant difference in cell surface expression between the two timepoints was recorded. These data suggest, that the human and porcine CMV-derived glycoprotein UL-16 partially retains pULBP1 intracellularly.

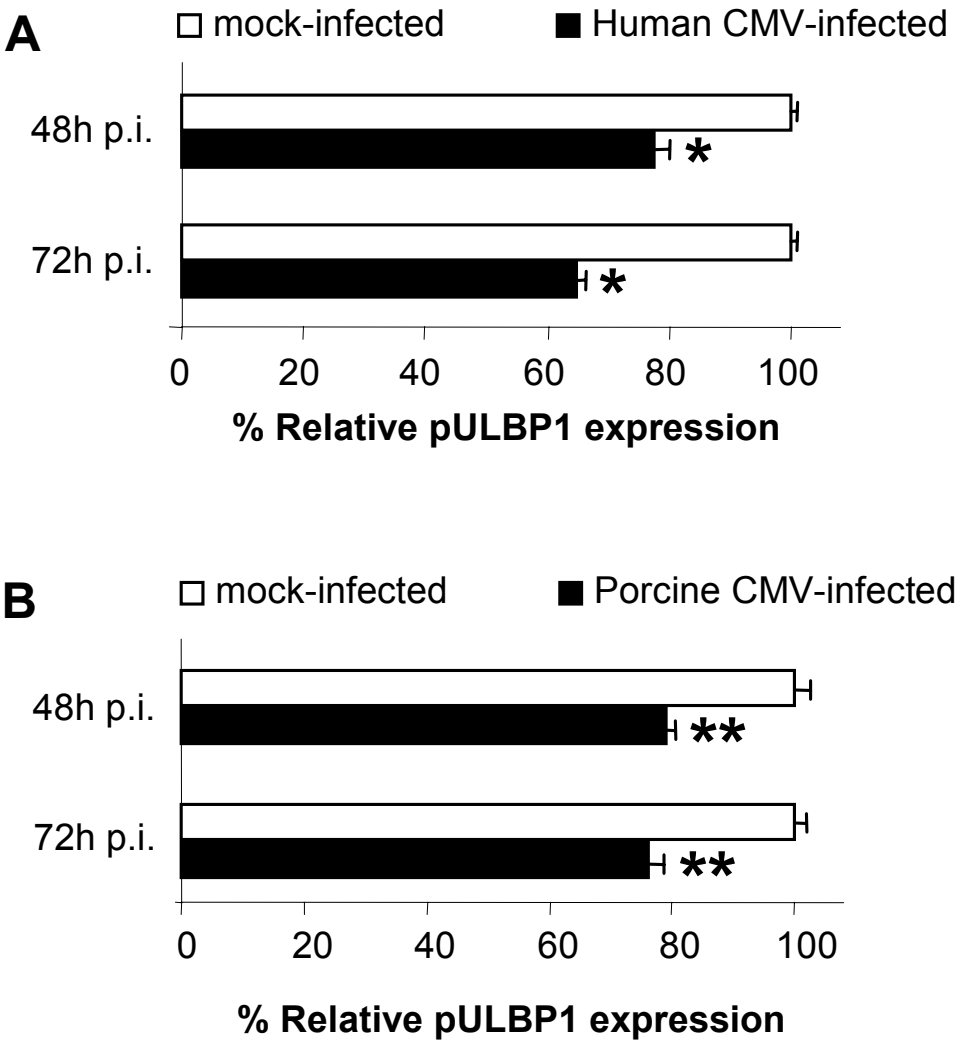

Figure 5: Human and porcine CMV infection decreases pULBP1 cell surface expression on primary PAEC. Shown is the relative percentage of pULBP1 cell surface expression after human CMV (A) and porcine CMV (B) infection as compared to mock-infected cells (index=100). Error bars represent SEM. Asterisks depict statistical significance in a student's t-test (*$p<0.0005$, $n=12$; **$p<0.0001$, $n=12$).

5.3.5. Discussion

NK cell-mediated rejection mechanisms may represent one of the remaining obstacles preventing the clinical application of pig-to-human xenotransplantation. Human NK cytotoxicity against pEC might depend on incompatible cross-species interactions between porcine MHC class I molecules and inhibitory human NK receptors (72) and simultaneously on recognition of putative porcine ligands by activating human NK receptors. Recently, we demonstrated that lysis of pEC mediated by freshly isolated human NK cells is mainly triggered by NKG2D (81) and that pULBP1 is the predominant, if not only, functional ligand for human NKG2D on porcine cells (157). Therefore, we generated a mAb against pULBP1 in order to

systematically test the influence of various stimuli on cell surface expression of pULBP1 on primary PAEC.

In this study we show that the mouse anti-pULBP1 mAb clone aE5-63 specifically binds pULBP1 on porcine cells. Unexpectedly, the anti-pULBP1 mAb only stained PFA-fixed but not unfixed cells. Since the mice were immunized with the pEC line PEDSV.15 it was expected to obtain a mAb that recognizes the native form of the protein. However, it is known that fixation and therefore denaturation might ameliorate the accessibility of certain epitopes by eliminating possible steric hindrance.

Both human and porcine TNF-α stimulation significantly downregulated pULBP1 cell surface expression on primary PAEC. It was previously published that human TNF-α is crossreactive with the porcine TNF-α receptor, inducing upregulation of SLA class I and class II, and of B7, vascular cell adhesion molecule (VCAM), and E-selectin (198). Therefore, TNF-α acts as a proinflammatory cytokine on PAEC and is likely to enhance the cellular response to xenogeneic organs in vivo. Since a reduced cell surface expression of pULBP1 leads to decreased NK cell susceptibility (157), TNF-α downregulation of pULBP1 may render pEC less susceptible to NK cytotoxicity. This might prevent pEC from being lysed after NK cell adherence and therefore contribute to increased infiltration of NK cells into the tissue.

The strong increase in pULBP1 cell surface expression on αGAL-positive PAEC upon coculture of primary with human serum was dependent on the presence of anti-αGAL Ab. In agreement, no significant increase in pULBP1 cell surface expression was observed on primary PAEÇαGAL KO when cocultured with human serum containing anti-αGAL Ab. Thus, the binding of αGAL-specific xenoreactive Ab may, in addition to the induction of hyperacute rejection, also stimulate innate immunity via pULBP1 cell surface upregulation. In this view, αGAL KO pigs may not only avoid hyperacute rejection, but also be less sensitive to human NK cell responses *in vivo*.

An earlier study showed a slight reduction of susceptibility of human microvascular EC to NK cytotoxicity after hypoxia/reoxygenation (199). In theory, this reduction may have been observed because of a slight reduction of one of the ligands for NK

activating receptors. However, our finding that pULBP1 expression was upregulated after hypoxia/reoxygenation does not support such an hypothesis. Nevertheless, Maurus et al. used human EC that express many different ligands for activating NK receptors, whereas pEC only express ligand(s) for NKp44 and pULBP1.

Herpesviruses, such as CMV, are associated with significant morbidity after solid-organ transplantation (97). In pig-to-baboon xenotransplantation, activation of porcine CMV has been associated with xenograft injury and an increased incidence of consumptive coagulopathy and graft loss (196). *In vitro*, infection of pEC with human CMV and porcine CMV led to a decreased pULBP1 cell surface expression. This finding is in line with results observed after the human CMV infection of human fibroblasts (194) and is possibly due to a, at least partial, intracellular retention of pULBP1 by the human CMV-derived glycoptotein UL-16. The exact mechanism underlying the data observed in this study is a topic of ongoing research. If the slight reduction of pULBP1 cell surface expression after porcine CMV infection is also due to a UL-16-like pCMV-derived protein remains unclear.

In conclusion, the monoclonal anti-pULBP1 Ab described in this study might represent a useful tool for NK-related research in the field of xenotransplantation. Furthermore, insights in the regulation of pULBP1 cell-surface expression might help to better understand the rejection mechanisms mediated by human NK cells in pig-to-human xenotransplantation.

5.3.6. Acknowledgements

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5.4. Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human NK-mediated cytotoxicity

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Xenotransplantation, in press

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Running title: HLA-E-mediated endothelial cell protection

Key words: NK cells, HLA-E, Cytotoxicity, CD94/NKG2A

Abbreviations: Ab: antibody; ADCC: Ab-dependent cell-mediated cytotoxicity; β2m: β2-microglobulin; ILT2: Ig-like transcript 2; KIR: killer immunoglobulin-like receptors; mAb: monoclonal Ab; MFIR: geometric mean fluorescence intensity ratio NCR: natural cytotoxicity receptors; NK cell: Natural killer cell; pEC: porcine endothelial cells; SCT: single chain trimer

5.4.1. Abstract:

Background: The susceptibility of porcine endothelial cells (pEC) to human natural killer (NK) cells is related to the failure of human MHC-specific killer inhibitory receptors to recognize porcine MHC class I molecules. The aims of this study were (1) to assess the protection of pEC against xenogeneic NK-mediated cytotoxicity afforded by the stable expression of HLA-E single chain trimers (SCT) composed of a canonical HLA-E binding peptide antigen, VMAPRTLIL, the mature human β2-microglobulin, and the mature HLA-E heavy chain, and (2) to test whether HLA-E expression on pEC and porcine lymphoblastoid cells affects the adhesion of human NK cells.

Methods: Porcine EC lines expressing different levels of HLA-E SCT were generated by Ca_2PO_4-transfection followed by limiting dilution cloning. Surface expression of HLA-E was measured by flow cytometry. Susceptibility of transfected pEC lines against human NK cells was tested in ^{51}Cr-release cytotoxicity assays. Interactions between human NK cells and HLA-E positive pEC or porcine lymphoblastoid cells were further addressed in adhesion and conjugation assays.

Results: The level of protection of pEC from human NK-mediated cytotoxicity correlated with the intensity of surface HLA-E expression. Furthermore, the HLA-E SCT-mediated protection was specifically reversed by blocking the HLA-E specific NK inhibitory receptor CD94/NKG2A. HLA-E expression does neither affect the adhesion of human NK cells to pEC nor the heteroconjugate formation between human NK and porcine 13271.10 cells.

Conclusions: Stable surface expression of HLA-E on pEC was achieved in the absence of extrinsic peptide pulsing and provided partial protection from human NK cytotoxicity. Though insufficient to inhibit xenogeneic NK cell reactivity completely, transgenic HLA-E expression on pig organs might contribute to a successful application of clinical xenotransplantation in combination with other protective strategies.

5.4.2. Introduction

In addition to overcoming hyperacute humoral rejection, cellular rejection mechanisms seem to play a crucial role to achieve prolonged graft survival in pig-to-

human xenotransplantation models (6). Among other cell types human NK cells may play an important role in the cellular rejection of porcine xenografts (46, 47). This hypothesis is supported by the finding that *in vitro* NK cells activate porcine endothelium upon direct contact and act as a potent source for pro-inflammatory cytokines such as interferon-γ (48). Human NK cells have further been demonstrated to adhere to and lyse porcine target cells both directly and, in the presence of human serum containing xenoreactive antibodies (Ab), by Ab-dependent cell-mediated cytotoxicity (ADCC) (54). In addition, pig organs perfused with human blood *ex vivo* were predominantly infiltrated by NK cells (57, 59), and NK cells were present in histological samples of graft rejection in concordant and discordant rodent and preclinical pig-to-baboon models (53, 60-62).

A variety of molecules that deliver either activating or inhibitory signals tightly regulate NK cell activity (200). There are four main activating NK receptors, including NKp30, NKp44, and NKp46, collectively named natural cytotoxicity receptors (NCR) (119), and NKG2D (120). As previously reported, human NK cells kill porcine endothelial cells (pEC) only through NKG2D and NKp44 receptors, whereas NKp30 and NKp46 do not play a role in NK-mediated xenogeneic cytotoxicity (81, 157). The positive signals transduced by activating NK receptors are balanced by several groups of inhibitory NK receptors that bind HLA class I molecules including killer immunoglobulin-like receptors (KIR), Ig-like transcript 2 (ILT2), and the CD94/NKG2 family (116). Consequently, NK cytotoxicity occurs if stimulatory signals outweigh inhibitory signals derived from a potential target cell. Porcine EC are susceptible to human NK-mediated lysis possibly due to the inability of their MHC class I molecules to signal through human NK cell inhibitory receptors (72).

One of the inhibitory human NK receptors, the CD94/NKG2A heterodimer, is widely expressed on human NK cells and binds HLA-E, a monomorphic MHC class I molecule expressed at weak levels on most tissues (136-138). We and others have previously demonstrated that the expression of human MHC class I molecules, including HLA-B27, -Cw3, -Cw4, -G, and –E, provides only partial protection against xenogeneic human NK cytotoxicity (74-79, 110). However, in these studies stable expression of HLA-E molecules was only achieved by the extrinsic pulsing with stabilizing HLA-E binding peptides and interferon-γ treatment (110). Although not

rigorously examined in the latter study, human β2-microglobulin (β2m) may also be required for optimal cell-surface expression of HLA-E in pig cells. Generating pigs transgenic for HLA-E, the final goal of this approach to protect porcine grafts from human NK cytotoxicity, would therefore require the addition of three human genes (HLA-E heavy chain, human β2m, and a gene encoding for an HLA-E binding peptide) in order to ensure stable HLA-E cell-surface expression. To circumvent this technically difficult and tedious obstacle, a single chain trimer (SCT) of HLA-E has been constructed and expressed in pig cells (201). This HLA-E SCT construct is composed of linked sequences encoding for a HLA-Cw*03-derived canonical HLA-E binding peptide antigen, VMAPRTLIL, the mature human β2m, and the mature HLA-E heavy chain. The first aim of this study was to test whether HLA-E SCT expression protects pEC against xenogeneic human NK cytotoxicity mediated by primary IL-2 activated human NK cells derived from healthy donors as opposed to the immortalized NK clonal cell lines previously employed (201). The second question addressed in this report was whether HLA-E surface expression on pEC and porcine lymphoblastoid cells interferes with the adhesion of human NK cells. This issue arose based on previous reports that NK receptors, including KIR2DL1 and ILT-2, not only inhibit NK cytotoxicity but also regulate cell adhesion through their interaction with HLA-Cw4 and HLA-G, respectively (75, 202).

5.4.3. Material and Methods

Cells

The SV40-immortalized aortic pEC line PEDSV.15 was established and characterized in our laboratory (163), and the porcine AOC endothelial cell line was reported previously (203). The immortalized porcine lymphoblastoid cell line 13271.10 was a gift of G. Waneck (Massachusetts General Hospital, Boston, MA) (76, 164). The 13271.10-E/A2 cell line (110), the isolation of PBMC from healthy blood donors, purification of NK cells, and generation of polyclonal human NK cell populations have been described previously (74). After isolation, NK cells were activated by culture in AIM-V medium (Invitrogen, Basel, Switzerland) supplemented with 10% human plasma obtained from healthy donors, 300 U/ml of human IL-2 (Chiron, Emeryville, CA), 1 mM sodium pyruvate, 2 mM L-glutamine, essential amino acids (50x), non-essential amino acids (100x) (all Invitrogen), 1% penicillin/streptomycin (Gibco, Basel, Switzerland), and 20 mM HEPES (Invitrogen).

Transfection

PEDSV.15 and AOC cells were transfected with the HLA-E SCT construct (201) using Ca_2PO_4-transfection and selected using G418 at a concentration of 0.5 mg/ml (Life Technologies, Gaithersburg, MD). After antibiotic selection stably transfected cells expressing HLA-E were further purified by limiting dilution cloning.

Flow cytometry

Surface expression of CD94 and NKG2A on human NK cells was analyzed on a FACScan (Becton Dickinson, Basel, Switzerland) by indirect immunofluorescence using the primary mouse mAb DX22 (anti-CD94, IgG_1, L. Lanier, University of California, San Francisco, CA) or Z199 (anti-NKG2A, IgG_{2b}, Beckman Coulter, Nyon, Switzerland). HLA-E SCT expression on PEDV.15 and AOC cells was analyzed using the 3D12 mAb (anti-HLA-E, IgG_1, D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA), which recognizes both HLA-E free chain and HLA-E noncovalently associated with peptide antigen and β2m (204) in addition to HLA-E SCT (204). FITC-conjugated goat anti-mouse IgG Ab (Chemicon International, Dietikon, Switzerland) was used as a secondary reagent. Phenotypic analysis of NK cells was carried out by direct immunofluorescence as described previously (74). To compare the levels of surface expression, the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity (MFI) of staining with the mAb of interest with the MFI of the control mAb.

Cytotoxicity assays

The cytotoxic activity of IL-2-activated polyclonal human NK cells was tested in 4 h ^{51}Cr-release assays in serum-free AIM-V medium as described previously (166). For blocking studies, NK cells were preincubated for 30 min at 4°C with 10 μg/ml of anti-CD94 (DX22), anti-NKG2A (Z199) or isotype-control mAb MOPC21 (IgG_1, Sigma, Buchs, Switzerland) or MOPC141 (IgG_{2b}, Sigma). Monoclonal Ab were also present during the coincubation of target and effector cells at a concentration of 5 μg/ml. After incubation for 4 h at 37°C the assays were stopped, ^{51}Cr-release was analyzed on a gamma counter and the percentage of specific lysis was calculated.

Conjugation assays

NK and 13271.10 cells were labeled at room temperature for 5 min with 10 μM of the fluorescent cell linkers PKH67-GL and PKH26-GL, respectively (Sigma). The staining reaction was stopped by adding two volumes of FCS. Cells were washed three times, incubated at 37°C for 2 h to allow excess of fluorochrome to diffuse off the cells, and resuspended in cold AIM-V medium supplemented with 5% FCS. $1x10^5$ labeled NK cells were mixed with $2x10^5$ labeled target cells in a final volume of 0.2 ml in 6-ml polystyrene FACS tubes (Becton Dickinson), centrifuged at 1000 rpm for 3 min, and immediately incubated at 37°C for 1, 5, 10, or 15 min. Following incubation, tubes were placed on ice and cell pellets were carefully disrupted by gentle mixing, fixed by adding 1 ml of cold paraformaldehyde, and immediately analyzed on a FACScan. Double positive $PKH26^+PKH67^+$ events were defined as conjugates. Results are expressed as percentage of NK cells that formed conjugates with target cells. To perform antibody blocking experiments NK cells were preincubated for 30 min at 4°C with 10 μg/ml of the following mouse IgG_1 mAbs: HP-2/1 (anti-CD49d), 7E4 (anti-CD18) (both Beckman Coulter, Nyon, Switzerland), or an isotype control mAb (MOPC21).

Adhesion assay

Adhesion of human NK cells on PEDSV.15 and AOC cells was analyzed using a modified Stamper-Woodruff assay as previously described (39). Briefly, pEC were grown to confluency in a 30 mm culture dish within the limits of a circle of 20 mm in diameter administered with a non-toxic silicon oil coat (dimethylpolysiloxane; Sigma). The resulting monolayers were washed and overlayed with 100 μl of Weissmann-buffer containing IL-2-activated NK cells (10^7 cells/ml), glucose (5.55 mM, Sigma) and human albumin (5 mg/ml, Fluka, Deisenhofen, Germany). The dishes were either incubated statically or were rotated at 64 rpm in a prewarmed (37°C) horizontal shaker-incubator (Infors AG, Bottmingen, Switzerland). This rotation speed induces a continuous flow of NK cells on pEC monolayers, thus simulating the shear stress and contact times present in the postcapillary vascular system. After 10 min the assay was stopped by rapidly placing the dishes on ice and by prefixing the cells for 2 min with 1% paraformaldehyde (Sigma) in PBS. The monolayers were then gently washed, fixed for additional 15 minutes, and finally protected with a glass coverslip. For quantification, four fields of 0.16 mm^2 were defined at a distance of 0.6 cm from

the center of rotation and the number of adhering cells was counted by light microscopy.

5.4.4. Results

Analysis of HLA-E SCT expression on transfected porcine cells

The expression level of HLA-E SCT on both PEDSV.15 (Fig. 1A) and AOC cells (Fig. 1B) varied widely between different clones obtained by limiting dilution cloning. In order to test the effect of different levels of HLA-E SCT cell surface expression in the experiments described below we selected three clones with either no, low or high HLA-E cell surface expression (Fig. 1). The cells were stained by the HLA-E-specific mAb 3D12, but not by the HLA class I-specific mAb DX17 presumably due to conformational changes (data not shown) Neither untransfected nor mock-transfected PEDSV.15 cells were stained by the anti-HLA-E mAb 3D12. The expression levels of HLA-E SCT remained stable over time as tested at different time points of cell culture (data not shown).

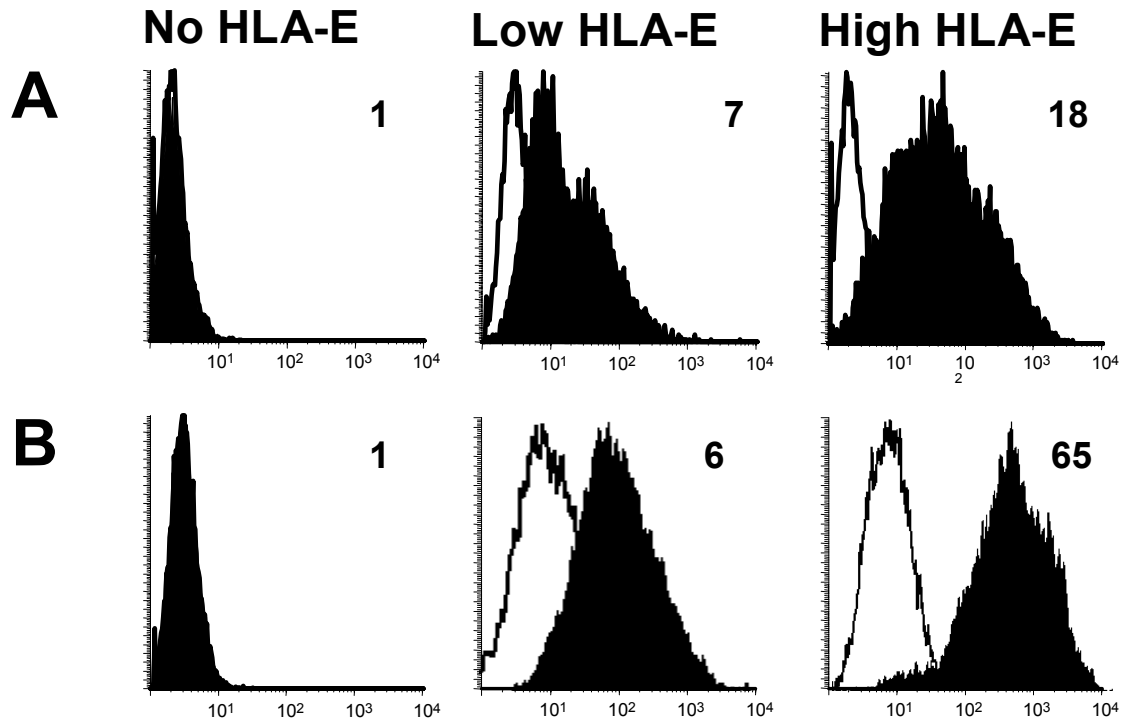

Figure 1: Cell surface expression of HLA-E SCT on transfected PEDSV.15 (A) and AOC (B) cells. Three selected clones for each cell line are depicted with either no (left panel), low (middle panel) or high (right panel) HLA-E cell surface expression. Cells were analyzed by indirect immunofluorescence staining using the HLA-E-specific 3D12 mAb (filled histograms). Histograms for an isotype-matched control mAb are also depicted (empty histograms). Numbers indicate the MFIR of HLA-E expression and are representative of at least two independent experiments.

HLA-E SCT expression protects porcine endothelial cells from xenogeneic human NK cytotoxicity

In order to test whether the HLA-E SCT-transfected pEC lines PEDSV.15 and AOC were protected from xenogeneic human NK cytotoxicity by HLA-E surface expression, we performed ^{51}Cr-release cytotoxicity assays using the different pEC clones described above as targets and IL-2-activated polyclonal human NK cell populations as effector cells. Since the protective potential of HLA-E depends on its recognition by the corresponding inhibitory NK receptor CD94/NKG2A, we also determined the CD94 as well as the NKG2A expression levels on the human NK cells used for xenogeneic killing assays by flow cytometry. HLA-E SCT protected pEC from human NK cytotoxicity to an extent dependant on both the level of surface HLA-E SCT expression on pEC and the level of CD94/NKG2A expression on human NK effector cells (Fig. 2). Pooled cytotoxicity results using human NK cells expressing different levels of CD94/NKG2A as effectors against PEDSV.15 and AOC target cells expressing low amounts of HLA-E SCT showed a mean reduction of cytotoxicity of 40±8% and 33±5% (±SEM; n=11), respectively. PEDSV.15 and AOC cells expressing high amounts of HLA-E SCT showed a reduced susceptibility against human NK cells of 48±5% and 45±8%. Taken together, the level of protection of pEC against human NK-mediated cytotoxicity depends on both the expression levels of HLA-E SCT and the respective NK receptor CD94/NKG2A.

Inhibition of NK-mediated cytotoxicity against HLA-E SCT-positive porcine endothelial cells is reversed by blocking of CD94/NKG2A

In order to confirm that the inhibitory effect observed in the previous experiments was indeed specifically mediated through the interaction of HLA-E SCT with CD94/NKG2A, we performed cytotoxicity assays in the presence of an anti-CD94 as well as an anti-NKG2A blocking mAb. NK-mediated cytotoxicity against pEC was completely restored by blocking CD94/NKG2A whereas isotypic control mAb had no effect (Fig. 3A). A general non-specific inhibitory effect of the anti-CD94/NKG2A mAb was excluded by their addition to HLA-E SCT-negative target cells (data not shown).

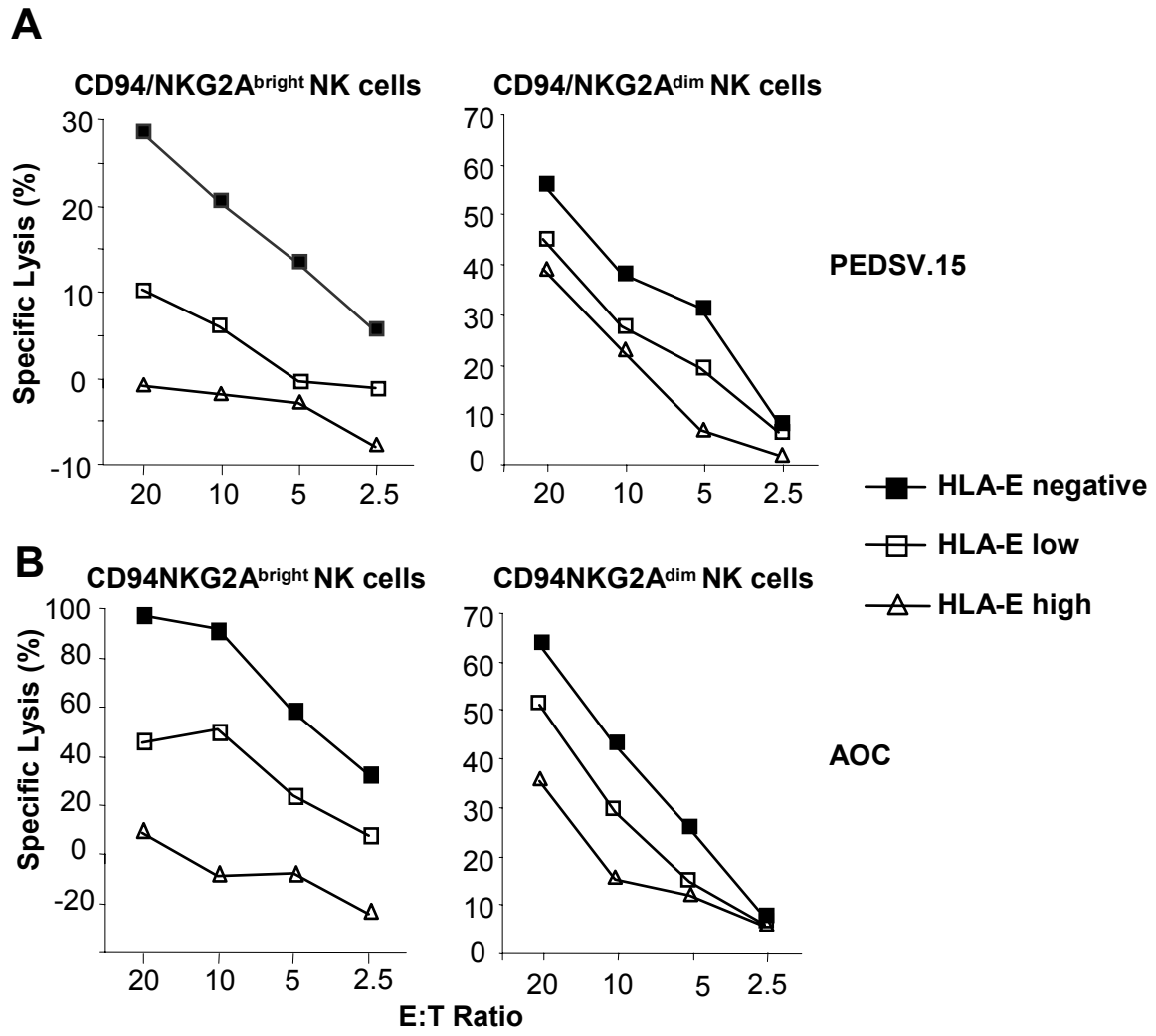

Figure 2: HLA-E SCT protects porcine endothelial cells from xenogeneic human NK cytotoxicity. Shown is the specific lysis obtained in 4 h 51Cr-release cytotoxicity assays using PEDSV.15 (A) and AOC (B) cells expressing different levels of HLA-E SCT as targets and either CD94/NKG2Abright (MFIR=34 and 9, respectively) or CD94/NKG2Adim (MFIR=7 and 2, respectively) polyclonal IL-2-activated human NK populations as effectors at four different E:T ratios. Results are representative for six independent experiments performed with NK cells purified from five different donors.

Pooled data of all cytotoxicity assays using either PEDSV.15 or AOC cells expressing low or high amounts of HLA-E SCT revealed an average reduction in NK-mediated cytotoxicity of 51±1% and 48±9%, respectively (Fig. 3B), with complete reversion by CD94-blocking. In conclusion, these data clearly show that the reduction of human NK-mediated cytotoxicity against HLA-E SCT positive pEC depends specifically on molecular interactions of HLA-E with CD94/NKG2A.

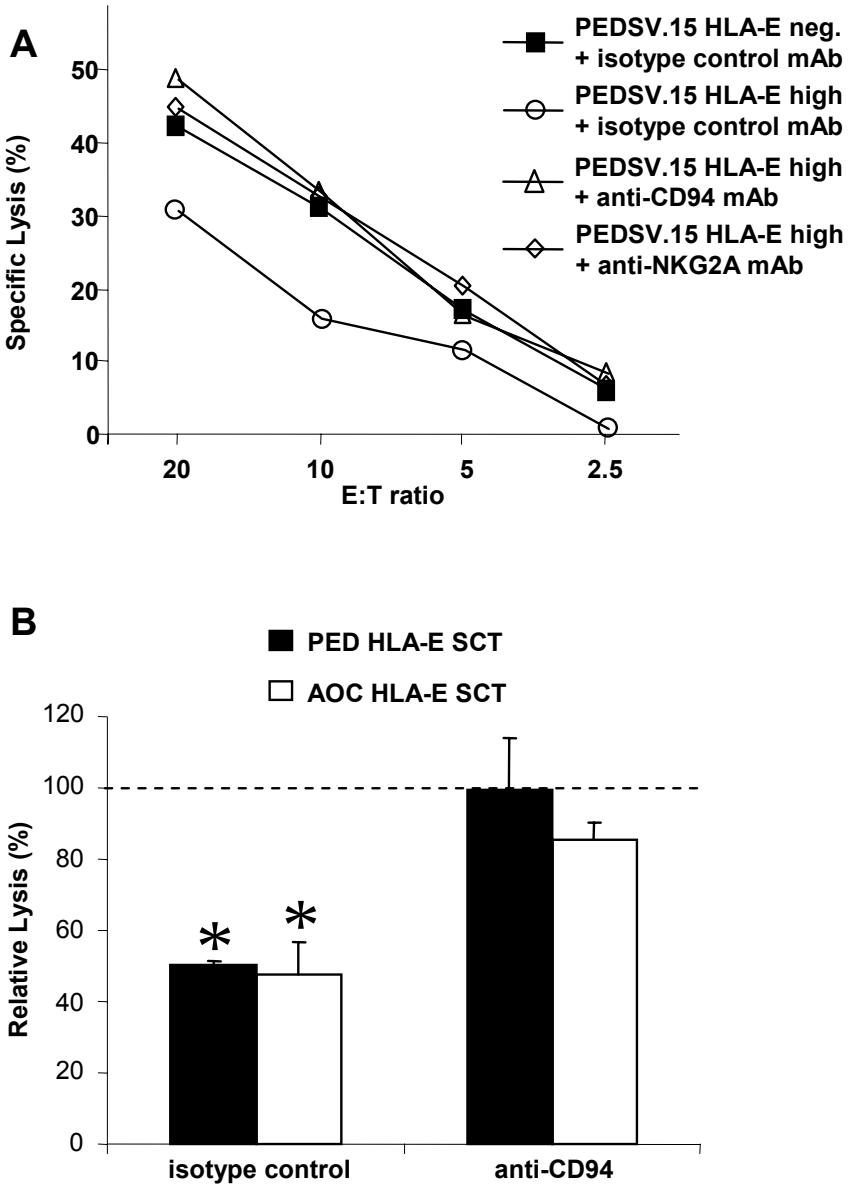

Figure 3: Inhibition of NK-mediated cytotoxicity against HLA-E SCT positive porcine endothelial cells is reversed by blocking of CD94/NKG2A. (A) Shown is a representative experiment where the cytotoxicity of IL-2-activated polyclonal human NK cells against PEDSV.15 cells negative for HLA-E SCT (closed symbols) and PEDSV.15 cells expressing high levels of HLA-E SCT (open symbols) was tested in 4 h 51Cr-release assays in the presence of anti-CD94 (triangles), anti-NKG2A (diamonds), or isotype-control (circles) mAb. (B) A summary of four independent experiments is shown. Cytotoxicity is expressed as percentage of relative lysis of PEDSV.15 (filled bars) and AOC (empty bars) cells expressing high levels of HLA-E SCT, in the presence of the indicated mAb, as compared to the respective intra-assay control (lysis of HLA-E SCT negative PEDSV.15 and AOC cells, respectively, dashed line, index=100). The mean relative cytotoxicity was calculated at three different E:T ratios (20:1 to 5:1), error bars indicate SEM. Asterisks designate statistical significance in a Student's t-test (*$p<0.01$). Dashed line marks 100% relative lysis.

HLA-E expression does not affect adhesion of NK cells to porcine endothelial cells or conjugate formation of NK cells with porcine lymphoblastoid cells

In order to test whether adhesion of human NK cells to pEC is reduced upon HLA-E SCT expression, a modified Stamper-Woodruff adhesion assay was performed either under static or under rotating condition. The number of CD94/NKG2A^{bright} NK cells

that adhered to pEC after 10 min co-incubation with a pEC monolayer was not influenced by the expression of HLA-E SCT (Fig. 4 A). To address the question whether HLA-E surface expression on porcine lymphoblastoid cells interferes with the adhesion of human NK cells, heteroconjugate formation between human NK and porcine 13271.10 cells was studied by flow cytometry. As previously shown, 13271.10-E/A2 cells stably express HLA-E without extrinsic peptide pulsing and are partially protected against human NK cytotoxicity (110). Binding equilibrium in the conjugation assay was reached by 10 min at 37°C with 49 ± 9% (n=6) of CD94/NKG2A^{bright} NK cells bound to 13271.10 cells. Conjugate formation was temperature-dependent, i.e. it was only observed at 37°C, but not at 4°C (data not shown), indicating that 13271.10 cells need to be metabolically active for conjugate formation or that membrane fluidity facilitating lipid raft migration and clustering are required to form the synapse. However, the expression of HLA-E on 13271.10 cells did not influence the number of conjugates formed with NK cells (Fig. 4B). To demonstrate that the formation of conjugates can be blocked, control experiments using mAb against integrins were performed. Conjugation between human NK cells and porcine 13271.10 cells was partially inhibited by masking the β_2-integrin CD18 but not the β_1-integrin CD49d (Fig. 4C), implying that β_2-integrin adhesion molecules are required for xenogeneic adhesion. In conclusion, these data strongly suggest that HLA-E expression does neither affect the adhesion of human NK cells to pEC nor the heteroconjugate formation between human NK and porcine 13271.10 cells.

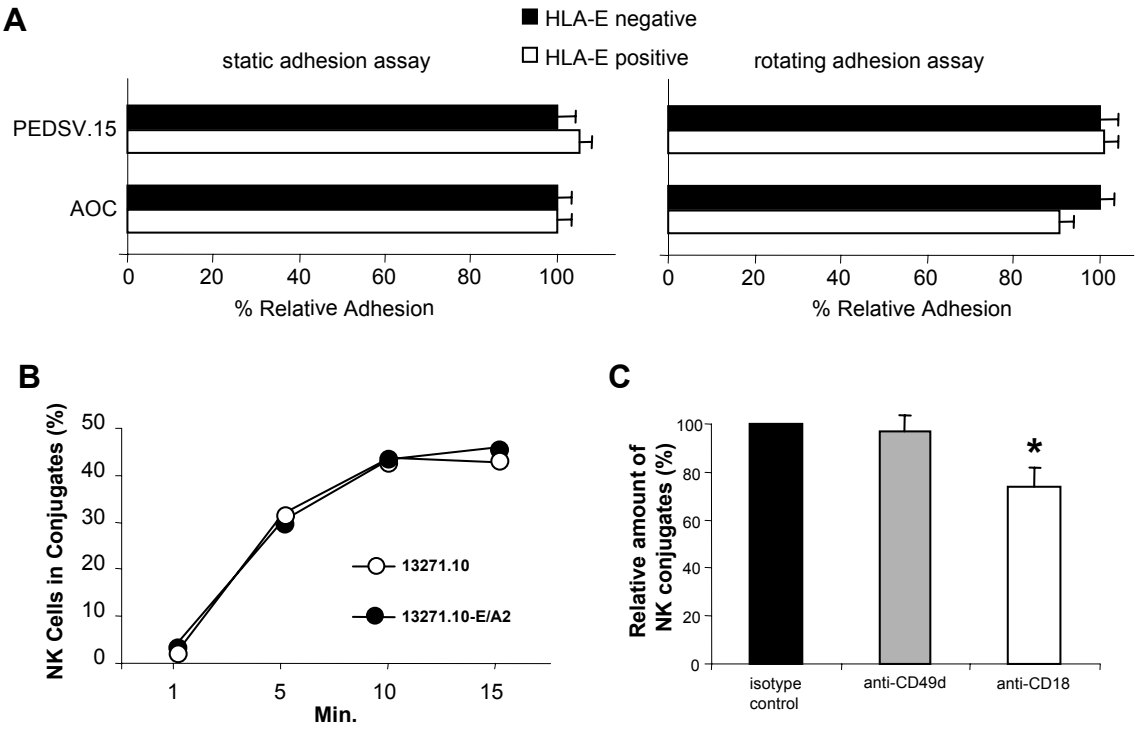

Figure 4: HLA-E neither affects the adhesion of human NK cells to pEC nor the conjugate formation between human NK and porcine 13271.10 cells. (A) Adhesion of human NK cells to pEC in a static and a rotating adhesion assay. Shown is the percentage of relative NK cell adhesion to HLA-E SCT positive (white bars) as compared to HLA-E SCT negative (black bars) PEDSV.15 and AOC cells, respectively. Error bars represent SEM. (B) The percentage of conjugates that NK cells form with 13271.10 (filled symbols) and 13271.10-E/A2 (open symbols) cells was measured at 37°C after 1, 5, 10, and 15 min incubation time. (C) Conjugate formation partially depends on the □□-integrin CD18. The percentage of conjugates between human NK cells and 13271.10 cells was measured after 10 min incubation at 37°C in the presence of an isotype control mAb (black bar), anti-CD49d mAb (gray bar), or anti-CD18 mAb (white bar). Shown is the relative amount of NK conjugates in the presence of the indicated mAb as compared to the percentage of conjugates in the presence of an isotype control mAb (index=100). The mean relative percentage of conjugates of three independent experiments is shown, as calculated from (5, 10, and 15 minutes). Error bars represent SEM. Asterisks mark statistical significance in a Student's t-test (*p<0.01).

5.4.5. Discussion

The non-classical human MHC molecule HLA-E is a potent inhibitory ligand for CD94/NKG2A receptor-bearing NK cells and, unlike classical MHC molecules, does not induce allogeneic T cell responses (79). Therefore, transgenic expression of HLA-E on pig organs has the potential for substantially alleviating human NK cell-mediated rejection of porcine xenografts without the risk of allogeneic responses. However, in order to express correctly folded HLA-E complexes on porcine cell surfaces, all three elements, the HLA-E heavy chain, human β2-microglobulin and a stabilizing peptide, may be necessary (110). It has been observed previously that transfection of a single chain trimeric HLA-E construct, consisting of all three elements in a single peptide chain, resulted in stable HLA-E surface expression on a porcine epithelial cell line and completely protected from xenogeneic NK cytotoxicity

mediated by monoclonal human NK lines (201). In this study, we observed a significant decrease of polyclonal human NK-mediated cytotoxicity against pEC by the transgenic expression of HLA-E SCT. However, the previous finding of complete protection against NK lines was not observed being in line with earlier observations using various other HLA constructs and polyclonal human NK cell lines as effectors (77, 110). In contrast to monoclonal NK lines such as NKL and NK92, primary human NK cells express a much broader and diverse repertoire of cell surface molecules potentially interacting with porcine target cells. The consequences might be either the lack of an inhibitory signal mediated by HLA-E SCT, or strong activating signals delivered to subsets of the polyclonal NK population. Diversity of the NK cell receptor repertoire is thought to be evolutionarily adaptive by not allowing a pathogen to completely shut off NK-mediated responses by interfering with just one molecular pathway. Moreover, it is well known that the polymorphism of MHC molecules in the population leads to differential responses of individuals to the same pathogens (205). The protective effect of HLA-E SCT expression on pEC against NK-mediated cytotoxicity was completely reversed by blocking CD94/NKG2A. This finding directly demonstrated the specificity of HLA-E SCT interactions with CD94/NKG2A. Moreover, the observation that CD94/NKG2A expression correlated with the inhibitory potential of HLA-E SCT is in line with earlier results (110), showing a clear correlation between CD94/NKG2A expression on NK clones and their cytotoxic potential against HLA-E-positive pEC. This fact supports the theory that the lack of complete protection from polyclonal NK populations is most likely explained by the presence of CD94/NKG2A-negative or dim effector subpopulations. Moreover, HLA-E is known to interact not only with the inhibitory receptor CD94/NKG2A but also with the activating receptor CD94/NKG2C. Although HLA-E might trigger an activating NK response our CD94- and NKG2A-blocking data indicate that HLA-E on pEC was solely interacting with CD94/NKG2A. In line with this observation, Valés-Gómez et al. reported that HLA-E loaded with the Cw*03 leader peptide, which was used for the HLA-E SCT construct in the present study, strongly binds to CD94/NKG2A but only poorly to CD94/NKG2C and therefore is unlikely to induce NK activation (206).

The NK effector cells used *in vitro* are highly activated by stimulation with IL-2. We assume that inhibitory HLA-E-specific signals are not sufficiently strong to override triggering signals delivered by activating receptors in the CD94/NKG2A dim NK cell

subset. Supporting this notion, a weak allogeneic NK-mediated cytotoxicity (10-20% specific lysis) can be observed using primary human target cells which are expected to express normal levels of HLA-E molecules (unpublished results). Moreover, using *ex vivo* naïve NK cells without *in vitro* culture/activation rather than activated NK cells revealed an even better relative protective effect of HLA-E expression in previously published xenogeneic NK cytotoxicity assays (77) as well as in our experiments (data not shown). Another possible explanation for the only partial inhibitory effect of HLA-E might be the upregulation of porcine ligands of other activating NK receptor such as NKG2D (150, 157) during cell culture adding to the overall triggering of NK cytotoxicity.

HLA-E expression on 13271.10 cells did not influence the conjugate formation with human NK cells indicating that CD94/NKG2A, unlike other NK receptors, is not involved in mechanisms that control cell adhesion. On the other hand, this finding suggests that the successful inhibitory interaction of HLA-E with CD94/NKG2A with respect to cytotoxicity is not explained by an inhibition of prior adhesion steps mediated by HLA-E.

In conclusion, high HLA-E expression on porcine cells achieved using a SCT construct prevents human NK cytotoxicity *in vitro* to a great extent. Unlike other approaches to express HLA-E on pig cells that depended on extracellular peptide loading, this construct might be used for the production of HLA-E transgenic pigs. Such pigs might eventually contribute to the successful future application of xenotransplantation in combination with other protective strategies.

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5.5. HLA-E/humanβ2-microglobulin transgenic pigs: Protection against xenogeneic human anti-pig natural killer cell cytotoxicity

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Author contributions: EHW designed, performed, and analyzed the experiments in figure 1 and 3. BGL designed, performed and analyzed the experiments in figure 4 and wrote most of the manuscript. SM, EM, and RS contributed to figure 1 and 3. NH and RW designed, performed, and analyzed the experiments in figure 2. BK and EW performed the *in vivo* work with the pigs. JDS contributed to experimental design paper writing.

manuscript submitted

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5.5.1. Introductory Paragraph

The development of α1,3 galactosyltransferase (α1,3GT) deficient pigs (101) was an important step to overcome hyperacute rejection of pig organs transplanted into primates (207, 208). However, the lack of galactose-α1,3-galactose (αGal) epitopes does not protect against subsequent rejection mechanisms, including xenogeneic natural killer (NK) cell responses (209). A majority of activated human NK cells express the HLA-E specific inhibitory receptor CD94/NKG2A. Based on *in vitro* results (110), the aim of the present study was to overcome xenogeneic NK cell responses by expressing functional HLA-E/humanβ2-microglobulin (huβ2m) in transgenic pigs. A transgenic founder animal (designated as #8404) with high copy number integration was obtained following microinjection and transfer of 1584 zygotes to 45 recipients. Presence of HLA-E/huβ2m was demonstrated by Northern, Southern, and Western blotting, immunohistochemistry and flow cytometry analyses. Finally, lymphoblasts and endothelial cells derived from these HLA-E/huβ2m transgenic pigs were effectively protected against human NK cell-mediated cytotoxicity. This novel approach against cell-mediated xenogeneic responses has major implications for the generation of multitransgenic pigs as organ donors for clinical xenotransplantation.

5.5.2. Results/Discussion

NK cells represent a potential hurdle to successful pig-to-human xenotransplantation since they infiltrate pig organs perfused with human blood *ex vivo* (57, 210) and lyse porcine cells *in vitro* both directly, and in the presence of human serum, by antibody-dependent cell-mediated cytotoxicity (ADCC) (211). NK cell autoreactivity is prevented by the expression of MHC class I ligands of inhibitory NK receptors such as HLA-E on normal autologous cells (212) including the HLA-E specific inhibitory receptor CD94/NKG2A that is expressed on a majority of activated human NK cells. It is generally believed that HLA-E, unlike classical MHC molecules, is not polymorphic and induces allogeneic T cell responses to a much lower degree (213). To overcome xenogeneic NK cell responses *in vivo* transgenic pigs expressing HLA-E/huβ2m were generated. HLA-E is assembled in the ER and transported to the cell surface as a stable trimeric complex consisting of the HLA-E heavy chain, β2m and a peptide derived from the leader sequence of some MHC class I molecules (214). Since the HLA-B7 signal sequence-derived peptide (VMAPRTVLL) has the strongest

effect on HLA-E cell surface expression (215), exon 1 coding for the signal peptide of a genomic 7.7-kb fragment of HLA-E (E*01033) (216) was replaced by exon 1 of HLA-B7 in the construct HLA-E^{sigB7} used for microinjection (Fig 1a). Functionality of the HLA-E^{sigB7} construct was tested *in vitro* by cytotoxicity assays using mock-transfected and HLA-E^{sigB7}-transfected K562 cells as targets for the human NK cell line NKL. HLA-E expression protected K562 cells against NK cell-mediated lysis by 50% (Fig 3a). To achieve appropriate cell surface expression of HLA-E in transgenic pigs, HLA-E^{sigB7} and a 15-kb genomic huβ2m fragment, were co-injected into pronuclei of zygotes. In total, 1584 microinjected zygotes were transferred to 45 recipient sows, six (13.3%) of which became pregnant. Two sows gave birth to a total number of nine piglets.

The presence of the transgenes was analyzed by PCR and Southern blot analysis of genomic DNA isolated from ear tissue or blood samples of the piglets identifying three male transgenic founder animals carrying both the HLA-E^{sigB7} and the huβ2m fragment. Pigs #8403 and #8405 revealed a low copy number integration, whereas a high copy number integration was detected in pig #8404 (Fig 1b). Northern blot analysis of peripheral blood mononuclear cells (PBMC) from founder #8404 revealed both HLA-E transcripts of 1.6 and 2.7 kb as expected due to differential polyadenylation (217) and a huβ2m-specific transcript of the expected size of 1.2 kb (Fig 1c). Gene expression of both transgenes was markedly lower in PBMC derived from founder #8405, and not detectable in founder #8403. As a positive control, the human melanoma cell line Mel-Juso was used. Similar results were obtained with RNA from human PMBC or different cell lines. Remarkably, both genomic copies and numbers of transcripts were higher in the founder animal #8404 as compared to cells and cell lines naturally expressing HLA-E and huβ2m. The number of integrated gene copies can explain the high expression of the transgenes. In line with the mRNA expression, flow cytometry analysis of founder #8404 PBMC and endothelial cells revealed high levels of stable HLA-E and huβ2m cell surface co-expression (Fig 1d and e). Correct peptide loading of HLA-E heavy chains in founder #8404 was indicated by Endopeptidase H-resistance in Western blot analysis of PMBC lysates (Fig 1f). Incubation with exogenous B7 peptide ligands did not further increase HLA-E cell surface expression on PMBC supporting the expression of functional HLA-E molecules (218) (data not shown). When founder #8404 was mated with wild-type

females hemizygous F1 offspring (e.g. #8568, #9427, #9432) was generated with similar transgene expression levels (data not shown).

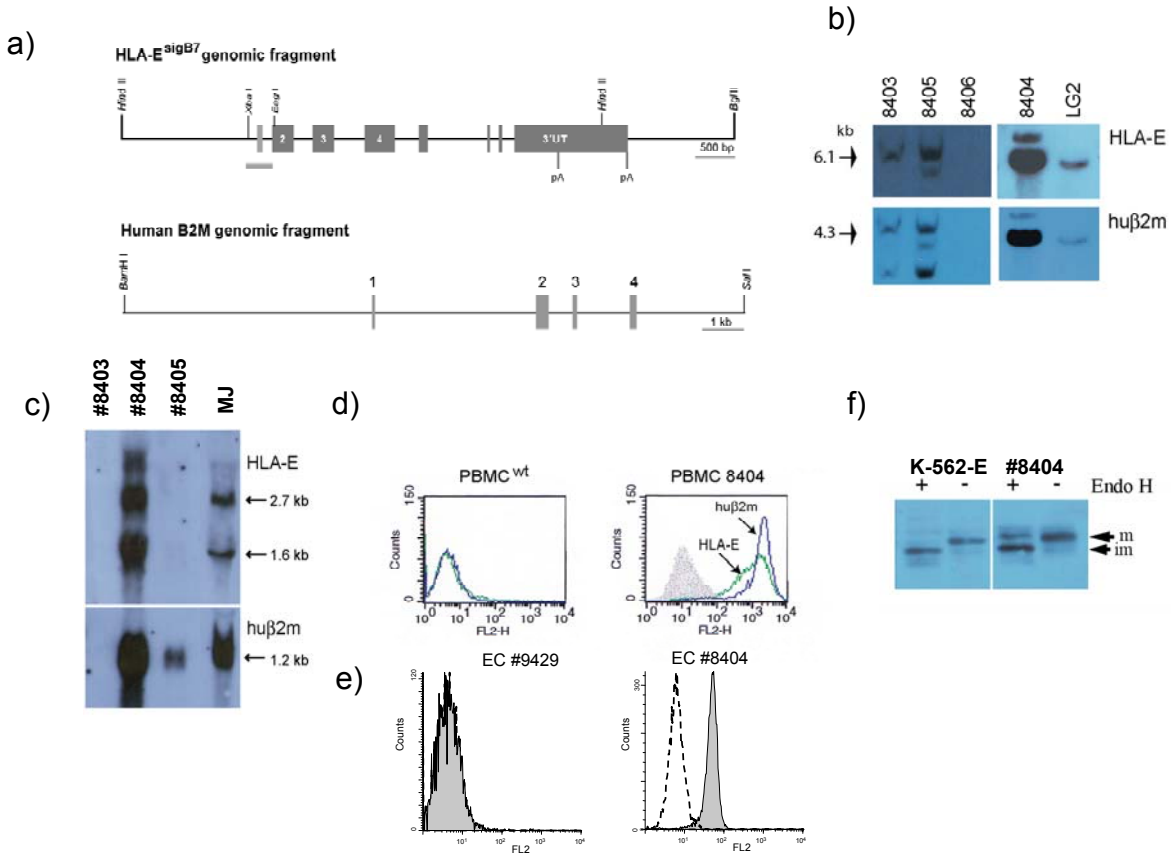

Figure1: a) The exon-intron organization of the microinjected 7.7 kb *Hin*d III/*Bgl* II fragment of HLA-E^{sigB7} and 15 kb *Bam*H I/*Sal* I fragment for huβ2m with relevant restriction sites are shown. The exchanged exon 1 to generate HLA-E^{sigB7} is underlined. Boxes depict exons and pA depicts the alternative polyadenylation sites in the 3´-untranslated region (3´-UT) of HLA-E. b) HLA-E and huβ2m genes are present in the genome of HLA-E/huβ2m-transgenic pigs. Shown is the Southern blot analysis of DNA derived from HLA-E/huβ2m-transgenic pigs designated as #8503 (low copy number), #8505 low copy number), #8406 (negative offspring), and #8404 (high copy number). DNA derived from B-LCL LG2 cells was used as positive control. c) HLA-E and huβ2m transcripts are present in PBMC derived from HLA-E/huβ2m-transgenic pigs. Shown is a northern blot analysis of RNA isolated from PHA-stimulated PMBC derived from HLA-E/huβ2m-transgenic pigs #8403, #8404, and #8405, and the human melanoma cell line MJ using the HLA-E 3´-probe (top) or the huβ2m cDNA (bottom). d) HLA-E and huβ2m are expressed on the cell surface of PMBC derived from HLA-E/huβ2m-transgenic pigs. PBMC derived from a wildtype control animal (left panel) and from the HLA-E/huβ2m-transgenic pig #8404 were stimulated for 48 h with PHA and subsequently analyzed for HLA-E heavy chain and huβ2m cell surface expression by flow cytometry. e) HLA-E is expressed on the cell surface of endothelial cells derived from HLA-E/huβ2m-transgenic pigs. Endothelial cells derived from a wildtype control animal (left panel) and from the HLA-E/huβ2m-transgenic pig #8404 were analyzed for HLA-E heavy chain cell surface expression by flow cytometry. f) Western blot analysis of cell lysates probed with the HLA-E specific mAb MEM E02 revealed correct peptide loading on HLA-E. K-562 HLA-E lysate was used as positive control, where due to inefficient ligand supply, HLA-E is not efficiently transported to the cell surface. Arrows mark the mature (m=Endo H resistant) and the immature (im=Endo H sensitive) HLA-E heavy chains.

Since the protective effect of HLA-E/huβ2m in pig-to-human xenotransplantation critically depends on the level and tissue distribution of transgene expression, protein

localization studies were performed by immunohistochemistry using different tissues. Although several different Ab either recognizing HLA-class I molecules (B9.12.1, A1.4) or against HLA-E (MEM E02) were tested, which detected HLA-E by FACS on transgenic PMBC or in cell lysates, it was not possible to demonstrate any specific HLA-E expression in transgenic pigs due to strong non-specific staining of porcine endothelial cells (pEC) in tissues from both wild-type and transgenic pigs. In contrast, immunostaining using the species-specific anti-huβ2m mAb BBM1 revealed high levels of specific expression in all transgenic pig tissues investigated. In the heart, expression of huβ2m was detected on pEC of capillaries and larger blood vessels (Fig. 2a). In the kidney, positive staining was observed both in glomerular and peritubular capillaries as well as on the endothelium of arteries and venous blood vessels of the cortex and medulla. Slight and diffuse positive staining was observed in the mesangium of glomeruli. Strong staining of the brush border and the apical part of proximal tubular epithelia was seen in a granular pattern (Fig. 2b). In the pancreas, capillaries and larger blood vessel pEC as well as epithelial cells of larger ducts stained positive for huβ2m (Fig. 2c). No positive staining for huβ2m was found in tissues of wild-type animals (data not shown).

The functional effect of HLA-E expression on PBMC derived from HLA-E transgenic animals in comparison to wild-type control animals was tested in NK cytotoxicity assays. As expected, the killing capacity of NK cells against HLA-E expressing target cells was significantly reduced, whereas wild-type target cells were readily lysed by IL-2-activated human NK cells (Fig 3b). However, only partial and not complete protection was observed, in particular at high, non-physiological E:T ratios. Since the protective effect of transgenic HLA-E expression is regarded to result from its interaction with the corresponding inhibitory NK receptor CD94/NKG2A, the remaining NK lysis is most likely explained by either CD94/NKG2A-independent cytotoxic mechanisms such as the Fas/FasL pathway and/or CD94/NKG2A-negative NK cell subsets present in the polyclonal effector population used in these killing experiments. To test the former hypothesis and to demonstrate that HLA-E transgenic lymphocytes are in general still susceptible to cell lysis, human 239 cells were transfected with the human CD95(Fas)ligand (293h95L) or mock-transfected (293pcDNA) and used as effector cells which induces apoptotic cell death by binding to CD95 on target cells (219).

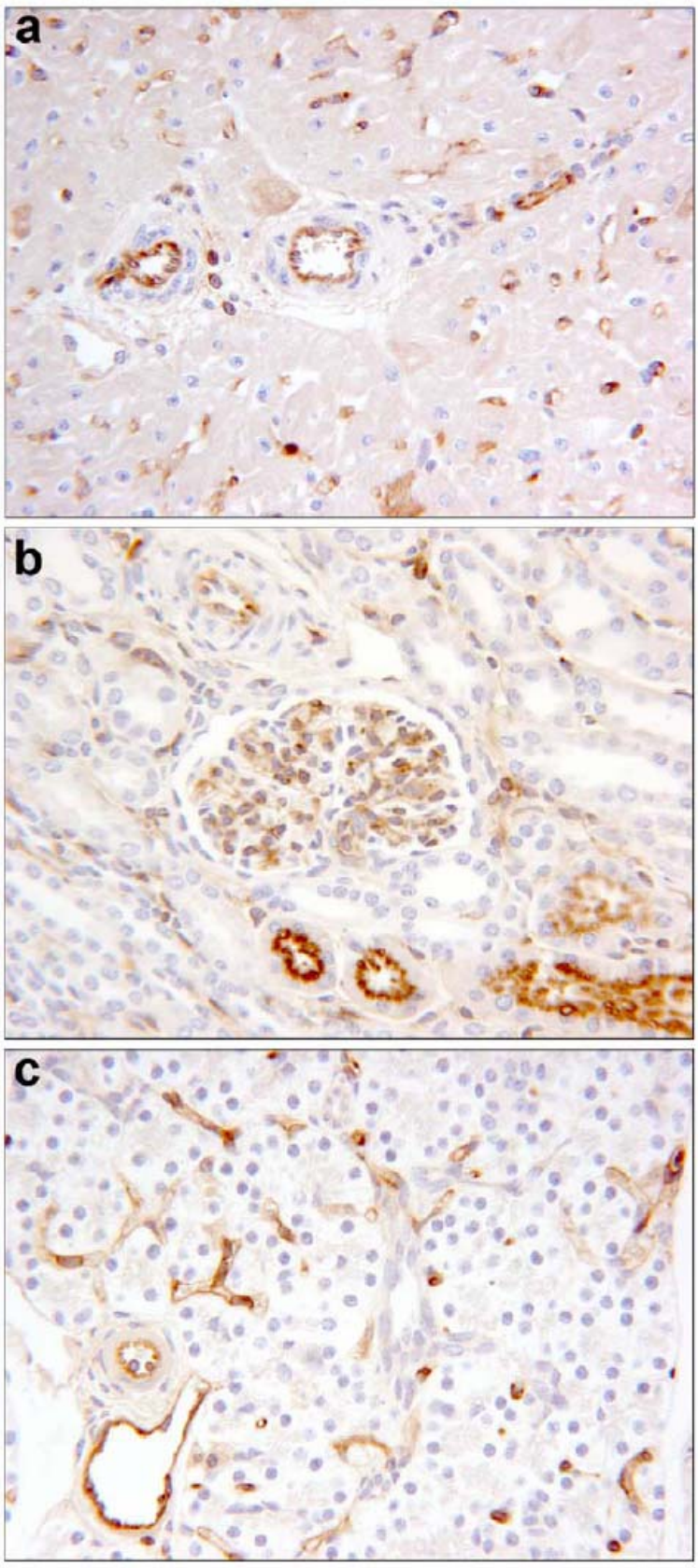

Figure 2: Immunohistochemistry revealed huβ2m expression on tissues derived from HLA-E/huβ2m-transgenic pigs. (a) Heart endothelia of capillaries and larger blood vessels stained positive for huβ2m. (b) In the kidney, positive staining was observed on endothelial cells of glomerular and peritubular capillaries as well as on endothelia of larger blood vessels, in the mesangium and, in a granular pattern, in the brush border and the apical part of proximal tubular epithelial cells. (c) Endothelial cells of pancreatic capillaries and blood vessels stained positive for huβ2m.

Whereas HLA-E transgenic porcine lymphocytes were partially protected against NK-mediated lysis, 293h95L effector cells were able to lyse HLA-E expressing as well as HLA-E negative control lymphocytes to the same extent at an E:T ratio of 10:1 (Fig 3c). These data show that HLA-E expression in transgenic pigs, at least on lymphocytes, does not interfere with the susceptibility to apoptosis mediated by the Fas/FasL pathway and suggests that the observed protection from NK lysis depends on inhibitory interactions between HLA-E and CD94/NKG2A.

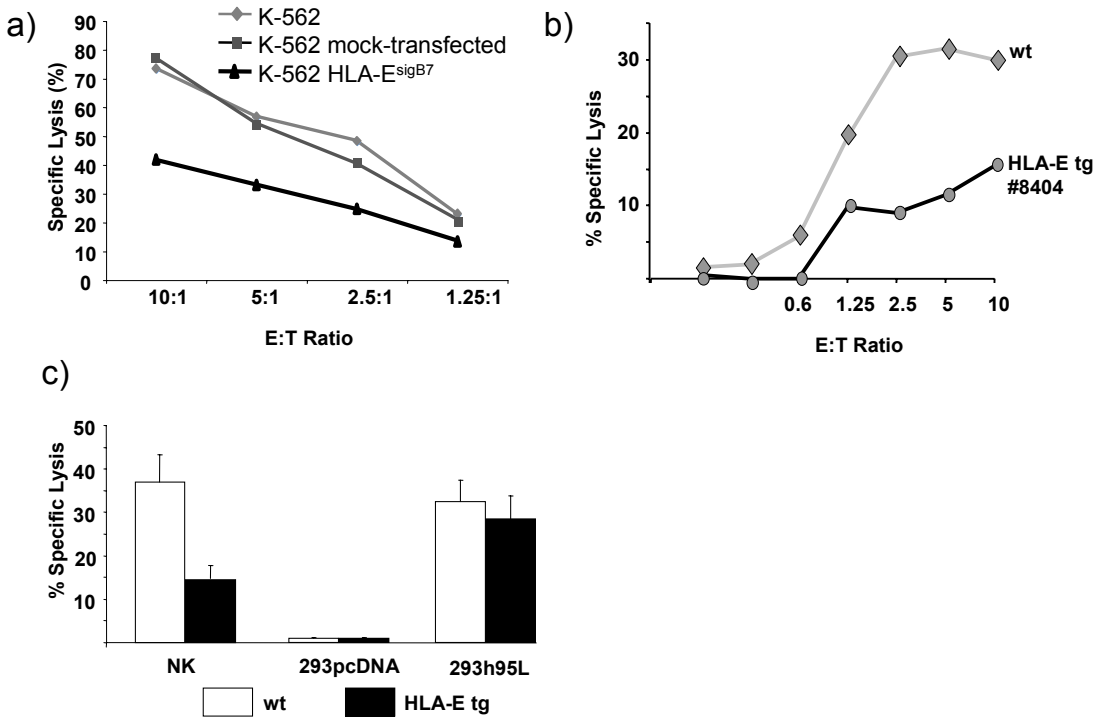

Figure 3: a) HLA-E^{sigB7} protects K-562 cells from NKL-mediated cytotoxicity. Shown is the specific lysis obtained in 4 h ^{51}Cr-release cytotoxicity assays using mock-transfected and HLA-E^{sigB7}-transfected K-562 cells as targets and the NK line NKL as effectors at different E:T ratios. b) Pig lymphoblasts derived from HLA-E/huβ2m-transgenic pigs are partially protected against human NK-mediated cytotoxicity. Shown is the specific lysis of either HLA-E negative (wt) or HLA-E positive (#8404) lymphoblast target cells by IL-2-activated human NK cells. Results are representative for three independent experiments. c) HLA-E expression does not influence CD95L-mediated cytotoxicity. 1 x 10^5 IL-2-activated human NK cells, 293pcDNA cells, or 293h95L cells were co-incubated with 10^4 ^{51}Cr-labeled lymphoblasts derived from the HLA-E/huβ2m-transgenic or a wild-type control pig. Bars represent percentage of specific lysis (mean $\pm$ standard deviation, n=3). Results are representative for two independent experiments.

Following pig-to-human xenotransplantation of vascularised solid organs the first porcine cell type in contact with human NK cells will be the pEC lining the capillaries and larger blood vessels. Therefore, three primary pEC were isolated from HLA-E transgenic pigs (#8404, #9427, and #9432, both offsprings of #8404) and a HLA-E negative litter mate(#9429), expanded *ex vivo* and tested for protection against

human NK cells lysis *in vitro*. Primary IL-2-activated human NK cells and the human NK line NK92 were used as effector cells. Furthermore, to demonstrate interactions between HLA-E and CD94/NKG2A, the expression of the latter on NK cells was measured by flow cytometry on all NK cells used in these assays. Surface expression levels of CD94/NKG2A varied considerably among different NK cell subsets, three of which were used for cytotoxicity experiments with either high (MFIR=72), medium (MFIR= 31), or low (MFIR=11) expression. As expected, the susceptibility of HLA-E positive targets correlated well with the expression of CD94/NKG2A on effector cells (Fig. 4a). The relative lysis of HLA-E positive target cells as compared to the negative control was 35±6% for CD94/NKG2A high NK cells, 62±6% for CD94/NKG2A medium NK cells, and 72±4% for CD94/NKG2A low NK cells. Blocking of CD94/NKG2A using a specific mAb rendered HLA-E positive target cells NK susceptible (Fig. 4b), further proofing functional specific interactions between HLA-E and CD94/NKG2A. Taken together, HLA-E positive pEC were partially protected from NK-mediated cytotoxicity by specific triggering of the inhibitory receptor CD94/NKG2A on human NK cells (Fig. 4c). Furthermore, *ex vivo* NK cell cytotoxicity using freshly isolated, naive human NK cells was almost completely inhibited even by CD94/NKG2A low NK cells (Fig. 4d). In addition, IFN-γ secretion of human NK cells after co-incubation with pEC was significantly reduced by the expression of HLA-E/huβ2m (Fig. 5). The partial protection of cytotoxicity and IFN-γ secretion is consistent with reports from the *in vitro* generation of pEC expressing HLA-E(78, 110, 201) and potential reasons might be miscellaneous. NK cells express a broad and diverse repertoire of cell surface molecules potentially interacting with porcine target cells. The consequences might be either the lack of an inhibitory signal mediated by HLA-E or strong activating signals delivered to subsets of the polyclonal NK population.

In conclusion, transgenic expression of HLA-E/huβ2m on porcine cells might contribute, in combination with other protective strategies, such as α1,3GT deficiency (101), and expression of human complement regulatory proteins CD46 (106), CD55 (107), or CD59 (108), to the successful prevention of xenograft rejection in clinical xenotransplantation.

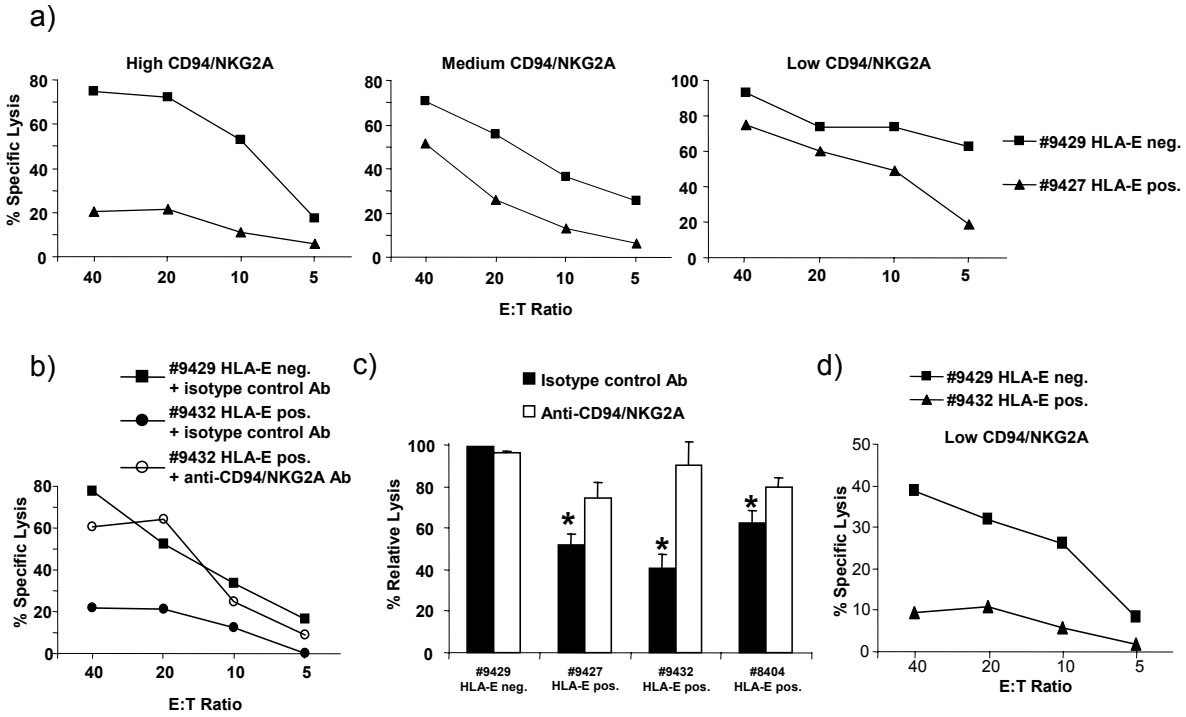

Figure 4: Susceptibility of aortic pEC derived from HLA-E/huβ2m-transgenic pigs against human NK cytotoxicity correlates with the expression of CD94/NKG2A on human NK cells and is reversed by anti-CD94/NKG2A blocking. (a) Shown is the percentage of specific lysis of either HLA-E negative (#9429) or positive (#9427) target cells by human NK cells expressing high, medium, or low amounts of CD94/NKG2A. (b) Inhibition of human NK cytotoxicity against HLA-E positive (#9432) pEC was blocked by anti-CD94/NKG2A mAb (open circle), while an isotype control mAb (closed symbols) had no effect. (c) A summary of two independent cytotoxicity assays with two different donors and the NK92 cell line is shown. As targets, pEC derived from three HLA-E positive pigs (#9427, #9432, and #8404) and an HLA-E negative litter-mate (#9429) were used. The mean relative cytotoxicity was calculated at four different E:T ratios (40:1 to 5:1), error bars represent SEM. Asterisks mark statistical significance in a paired student's t-test (*p<0.0001). (d) Shown is the percentage of specific lysis of either HLA-E negative (#9429) or positive (#9432) target cells by human NK cells expressing low amounts of CD94/NKG2A.

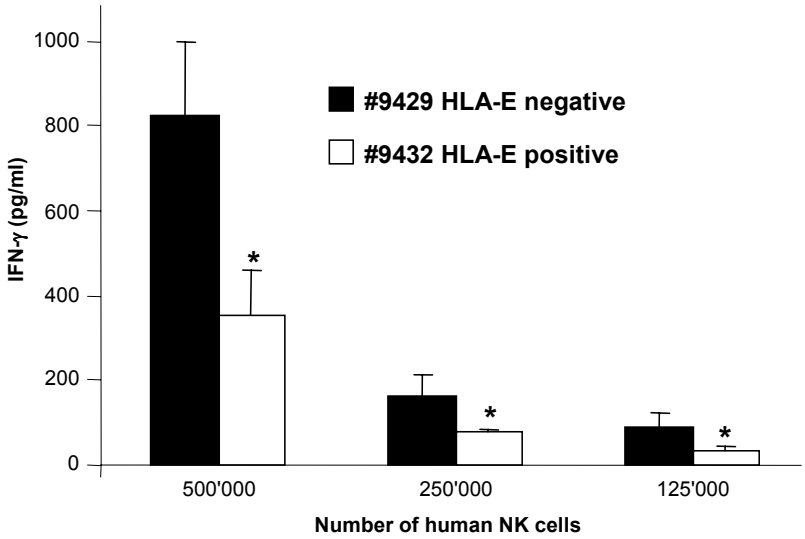

Figure 5: IFN-γ secretion of human NK cells after co-incubation with pEC is reduced by the expression of HLA-E/huβ2m. Shown is the amount of IFN-γ secreted by human NK cells after 48h of co-incubation with either HLA-E negative (#9429) or positive (#9432) pEC. Error bars represent SD. Asterisks mark statistical significance in a paired student's t-test (*p<0.05).

5.5.3. Materials and Methods

Construction of HLA-E^{sigB7}and huβ2m fragments

A genomic fragment of 330 bp encoding exon 1 of HLA-B7 was isolated by PCR using the primer pair B7-5´UT (5´-GATATCTAGAAGCCAATCAGCG-3) and Eex2as (5´-GCGGCCGGGCCGGGACACGGAAGTGTGGAAATACTTCAAGG-3) and was subcloned into the vector pCR®2.1-Topo ® (Invitrogen, Netherlands). Afterwards, it was inserted in the 7.7 kb genomic HLA-E Hind III/Bgl II fragment (HLA-E*01033)(220) using the Xba I and Eag I restriction sites. Cloning of huβ2m has been previously described(220). The fragments were isolated by restriction enzyme digestion and subsequent gel purification. Precipitated DNA was dissolved at 1000 copies/pL (500 copies of each gene) in 10 mM Tris/HCl, pH 7.5, 0.25 mM EDTA.

Generation of HLA-E/huβ2m transgenic pigs

Prepubertal gilts (German Landrace × Pietrain) were superovulated by intramuscular injections of 1200 IU equine chorionic gonadotropin and, 72 h later, 750 IU human chorionic gonadotropin (Intergonan® and Ovogest®; Intervet, Tönisvorst, Germany)(220). Twenty-four and 36 h after Ovogest® injection, donors were artificially inseminated with 3×10^9 sperms. Thirty-two to 34 h after the first insemination, donor gilts were sacrified, the oviducts were recovered and flushed with 20 ml of Dulbecco's phosphate-buffered saline (PBS) containing 5% heat-inactivated lamb serum (Gibco BRL) and 50 mg/L gentamicin sulfate (Sigma, St. Louis, MO). Zygotes were collected under a light microscope (Zeiss Stemi SV 6; Zeiss, Oberkochen, Germany) and centrifuged for 3 min at 15,000g to visualize the pronuclei. Microinjection was performed using an inverted microscope (Axiovert 135; Zeiss). After microinjection, embryos were stored in the incubator (Heraeus Holding GmbH, Munich, Germany) at 39°C, 5% CO_2, and 90% N_2 before the transfer. Endoscopic embryo transfer was performed the same day according to the procedure of Besenfelder et al.(221). Thirty to 35 injected embryos were inserted into one oviduct. Twenty to 25 days after the transfer, recipients were examined ultrasonographically for pregnancy. After farrowing, skin samples (ear tips) of the born piglets were taken for examination of transgene integration by PCR and Southern blot analysis.

Analysis of transgene presence by Southern, Northern, and Western blotting

Southern and Northern blot analyses were performed after a nonradioactive hybridization protocol(222) using the following probes. The HLA-E specific probe (HLA-E 3´-probe) encompassing exons 5 to 8 of the HLA-E transcript was obtained by RT-PCR using the primer pair (5´-CAGCATGAGGGGCTACCCG-3´) and (5´-GTGTGAGGAAGGGGGTCTG-3´) and was subcloned into pCR®2.1-TOPO ® (Invitrogen). This was the only HLA-E probe that did not show cross-hybridization with swine sequences. The huβ2m probe used for Northern blots was a full-length huβ2m cDNA clone whereas for Southern blots a genomic fragment of 500 bp containing exon 2, generated by PCR using the primer pair 2s (5´-CCCAAGTGAAATACCCTGGCA-3´) and 2as (5´-CGAAGATTCCCTGACAATCCC-3) was used.

Western blot analysis: Lysates equivalent to 100 µg of protein were subjected or not to 100 IUB mU of Endo Hf (New England Biolabs, USA) for 3 h at 37°C, separated by 10% SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Germany) using standard Western blotting techniques. HLA-E heavy chains were detected with the mAb MEM E02 (Acris, Germany) and the Western-Light chemiluminescence detection system according to manufacturer's recommendations (Invitrogen).

Immunohistochemistry

Immunohistochemistry for huβ2m and HLA-E was performed on formalin fixed, paraffin embedded heart, kidney and pancreas samples of HLA-E/ huβ2m-transgenic (n=2) and wild-type (n=2) pigs, using the streptavidin-biotin-method. The following Ab were used: undiluted mouse anti-huβ2m hybridoma supernatant (clone BBM1), mouse anti-human HLA-E (clone MEM E02)(223), and biotinylated goat anti-mouse Ig (both diluted 1:100 in tris buffered saline, pH 7.4). The indirect peroxidase and the streptavidin-biotin-method were applied on frozen tissue sections to stain for HLA-E, using the undiluted monomorphic hybridoma supernatant (clone B9.12.1) or A1.4 (Antigenix America, USA). Biotinylated goat anti-mouse Ig or horseradish peroxidase conjugated rabbit anti mouse Ig were diluted 1:100 and 1:300 in TBS, respectively. DAB was used as chromogen. Specificity controls included substitution of primary antisera with non-immune serum and omission of the secondary antiserum.

Cells

The primary porcine aortic endothelial cells were isolated as described earlier(163) and cultivated in RPMI 1640 (Invitrogen) supplemented with 20% FCS (PAA Laboratories, Luzern, Switzerland), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (100x), essential amino acids (50x), and 1% penicillin/streptomycin (all Invitrogen). The immortalized human NK line NK92 (a kind gift of C. Falk, gsf, Munich, Germany) was cultured in RPMI 1640 supplemented with 15% FCS, 5% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% penicillin/streptomycin. Isolation of PBMC from healthy blood donors, purification of NK cells, and generation of polyclonal human NK cell populations have been described previously(74). To obtain lymphoblasts, PMBC were stimulated with either 5 µg/ml PHA (Sigma Germany) for 48 hrs or with ConA (2 µg/ml, Pharmacia Germany) for 3 days. Isolated NK cells with a purity of routinely >95% were activated by culture in AIM-V medium (Invitrogen) supplemented with 10% human plasma obtained from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, essential amino acids (50x), non-essential amino acids (100x), 1% penicillin/streptomycin (Invitrogen), 20 mM HEPES, and 300 U/ml of human IL-2 (Chiron, Emeryville, CA). The melanoma cell line Mel-Juso and the lymphoblastoid B cell line LG2 obtained from J. Johnson LMU Munich, were cultured in RPMI medium, 1 mM sodium pyruvate, 1% penicillin/streptomycin (Invitrogen) supplemented with 10 % FCS (Biochrom, Germany)(217).

Flow cytometry

Cell surface expression of HLA-E und huβ2m was measured on a FACScan (Becton Dickinson, Mountain View, CA) by indirect immunofluorescence. After resuspension of $2x10^5$ cells per tube in staining buffer (HANKS, 0.1% BSA) cells were incubated for 30 min at 4°C with saturating amounts of Ab. As primary Ab the mouse mAb BBM1 (anti- huβ2m, ATCC, Wesel, Germany), TP25.99 (anti-HLA-E heavy chain, a kind gift of S. Ferrone, Roswell Park Cancer Institute, Buffalo, USA), and 3D12 (D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) were used. As secondary reagents PE-conjugated goat F(ab')2 anti-mouse Ig (Dako, Germany) was used. Surface expression of CD94/NKG2A on human NK cells was analyzed on a FACScanto (Becton Dickinson, Basel, Switzerland) by indirect immunofluorescence. As primary Ab the mouse mAb DX22 (anti-CD94, IgG_1, L. Lanier, University of

California, San Francisco, CA) was used. As secondary reagent FITC-conjugated goat anti-mouse IgG Ab (Chemicon International, Dietikon, Switzerland) was used. Phenotypic analysis of NK cells was carried out by direct immunofluorescence using FITC-UCHT1 (anti-CD3), PE-B73.1 (anti-CD16), and PE-B159 (anti-CD56) mAb (all from Pharmingen, Allschwil, Switzerland). An irrelevant, isotype-matched control mAb (MOPC21, mouse IgG1, Sigma) was used as control for mAb stainings. To exclude dead cells, propidium iodide gating was performed in all experiments. To compare the levels of surface expression, the geometric mean fluorescence intensity ratios (MFIR) were calculated by dividing the mean fluorescence intensity of each sample with the mean fluorescence intensity of the control Ab.

Cytotoxicity assays

The cytotoxic activity of IL-2 activated human NK cells and the human NK lines NK92 and NKL was tested in 4 h ^{51}Cr-release assays in serum-free AIM-V medium as described previously (74). Briefly, labeled target cells were added to triplicate samples of serial twofold dilutions of NK cells in round-bottom 96-well plates. Various E:T ratios were used in each experiment. For blocking studies, NK cells were preincubated for 30 min at 4°C with 10 µg/ml of the following mAb: MOPC21, DX22. MAb were also present during the co-incubation of target and effector cells at a concentration of 5 µg/ml. After incubation for 4 h at 37°C, the assays were stopped, ^{51}Cr-release was analyzed on a gamma counter and the percentage of specific lysis was calculated.

IFN-γ ELISA

Freshly isolated human NK cells ($5x10^5$, $2.5x10^5$,$1.25x10^5$) and HLA-E/huβ2m negative or positive pEC ($5x10^4$) were co-cultured in 200 μl AIM-V for 48 hours at which time 100 μl supernatant was removed and assayed for IFN-γ using an ELISA kit according to the protocol recommended by the supplier (Mabtech AB, Hamburg, Germany).

5.5.4. Acknowledgements

Stefanie Faerberboeck is kindly acknowledged for technical assistance.

6. Discussion

The role of NK cells in solid organ rejection is controversial. NK cells are thought to play a more pronounced role in xenotransplantation as compared to allotransplantation. However, recent findings suggest that the role of NK cells in solid organ allograft rejection needs to be reassessed (47). This is supported by the facts that NK cells (i) are able to reject hematopoietic and tumor cells, (ii) react to allogeneic MHC class I molecules, and (iii) possess the necessary effector mechanisms to induce cell death both directly and indirectly. In several studies NK depletion did not lead to a prolonged graft survival, which was interpreted that NK cells do not play a role at all in the process of rejection. But although NK cells might not be sufficient to reject a solid allograft alone or directly, they may participate in an acute rejection response by facilitating the actions of alloreactive T cells. In agreement, Maier et al. observed a significantly prolonged allograft survival in T cell costimulation impaired $CD28^{-/-}$ mice, as compared to wild type as well as NK-depleted wild type mice (224). Other studies have shown the presence of activated NK cells following infiltration into solid organ allografts which might at least amplify intragraft inflammation (225). NK cells also have the ability to interact with cells of the adaptive immune system and therefore serve as a bridge between innate and adaptive immunity. One example is the discovery that NK cells can promote alloreactive CD4+ T cells to differentiate along a Th1 pathway (226). NK cells also support the maturation of immature dendritic cells (227) and produce IFN-γ that may contribute to the alloresponse by up-regulating MHC class II expression on graft EC (228). Only few studies investigated the role of NK cells in human transplantation and conflicting results were observed. NK cells infiltrate human renal allografts during acute rejection and express cytolytic proteins such as granzyme A and B (229, 230). Furthermore, Vampa et al. noted a high correlation between the presence of predicted NK cell alloreactivity and the ability of these NK cells to mount a donor-specific cytotoxicity (231). However, no such correlation was found by Oertel et al. (232).

Focusing on xenotransplantation, NK cells appear to play an important role in the rejection of solid organ xenografts (46, 54, 233). This observation was made *in vitro* as well as *in vivo* in human (57, 58), nonhuman primates (60), and rodent systems

(234, 235). Moreover, NK cell rejection is important in hematopoietic stem cell xenotransplantation, and has been proposed as a method to induce xenogeneic tolerance (190). Importantly, NK cells promote xenograft rejection through several separate pathways: Firstly, NK inhibitory receptors fail to recognize pig MHC class I (72). Secondly, NK cells lyse porcine cells in an Ab-dependent manner by ADCC, induced by xenoreactive Nab bound to pEC (54). Thirdly, the NK activating receptors NKG2D and NKp44 recognize corresponding ligands on porcine cells (81, 157). And lastly, NK cells activate pig endothelium resulting in an induction of procoagulant factors and adhesion molecules that promote immune cell invasion (18, 19).

In summary, a growing number of studies, both *in vitro* and *in vivo*, describe the involvement and importance of NK cells in allotransplantation and, to an even greater extent, in xenotransplantation. Therefore, research aiming at identifying mechanisms of detrimental NK effects, might have a significant impact on the outcome of xenotransplantation. However, a future advent of clinical xenotransplantation relies on a simultaneous interference with several pathways, including such related to NK cells as well as to other leukocyte subsets.

In this thesis, mechanisms mediating activation and inhibition of human NK cells were studied in a pig-to-human xenogeneic setting. A common criticism of these studies was the strict use of *in vitro* models. Of course it is not possible to mimic all the dynamic processes of the immune system *in vitro*. On the other hand, most *in vivo* models are also highly artificial using inbred animal strains with homologous MHC haplotypes, hosted under sterile conditions. In general, *in vitro* studies allow distinct investigations under well-controlled conditions, while *in vivo* studies permit analysis under accurate physiological conditions where, however, all the factors involved are much more difficult to control and examine. Still, the studies described herein used EC, the first immunological target following vascularized pig-to-human xenotransplantation, as target cells in the ^{51}Cr-release assays. Furthermore, both immortalized and primary pEC were used as targets. The conclusiveness of the current studies is also improved by the use of both freshly isolated and cultivated primary human NK cell populations, individually derived from a large donor pool. A concern, however, are the E:T ratios of the cytotoxic assays which may be unphysiologically high. Nevertheless, the cocultivation time *in vitro* was limited to 4 h,

whereas *in vivo* every EC may encounter a large number of NK cells sequentially, resulting in an accumulation of damages over time.

Intriguingly, the significant variability among donors in the response of freshly isolated NK cells against pEC did not correlate with the NKG2D receptor surface densities, which were similar between different donors. Therefore, the reason for this variability might depend on individual differences in the efficiency of the respective cytotoxic effector pathways, such as perforin-granzyme release which is important in xenogeneic NK cytotoxicity (168). Indeed, it has been shown that NK cell culture conditions, in particular IL-2 and IL-15 supplements, upregulate the expression levels of molecules involved in the perforin-granzyme cytolytic pathway (169). Moreover, the cytolytic potential of individual freshly isolated NK populations might depend on the immunologic condition of the donor. In this respect, NK cells collected at different time points from the same donor differed in their ability to kill pEC without apparent differences in NKG2D expression (own unpublished observations). Taking the limitations of *in vitro* studies into careful consideration, NK cytotoxicity studies may represent relevant NK cell responses *in vivo*. Nevertheless, complementary *in vivo* experiments would add additional insights into the results and conclusions of the current study.

The involvement of NKp44 and NKG2D in NK-mediated lysis of pEC indicates the presence and recognition on pEC of porcine homologues of human NKp44 and NKG2D ligands or of "unique" ligands in pigs without apparent human homologues. Xenogeneic compatibility between NK activating receptors and their respective ligands has also been described in other species combinations. In fact, the putative ligand for the human NKp46 receptor is responsible for xenogeneic NK-mediated lysis of murine cells (170). The notion that NCR and NKG2D receptors are, at least partially, conserved between humans and other species is clearly different from the divergent evolution of the MHC class I-specific inhibitory receptors (171-173). Allelic specificity of human KIR for HLA class I molecules suggests a coevolution of inhibitory KIR with the respective MHC genes. This may explain why human KIR have a low affinity for MHC class I molecules of unrelated species (174). In contrast, it is conceivable that NCR and NKG2D as well as their ligands have not been

subjected to the pressure that caused the evolution of MHC genes and their receptors.

Beside the fact that pULBP1 is the predominant, if not the only, functional porcine ligand for human NKG2D, pEC express at least one alternative ULBP-like transcript, the pULBP2. However, the mRNA expression level of pULBP2 is about 20-fold lower than pULBP1. Also, preliminary analysis of a porcine bacterial artificial chromosome clone suggests several additional loci encoding ULBP-like proteins (M. Crew, unpublished observations). Thus, similar to humans, mice, cattle and primates, pigs may possess several ULBP and MIC proteins which serve as ligands for NKG2D (150, 151, 188, 189). The redundancy of the NKG2D system within a species might be driven by immune evasion mechanisms of pathogens such as CMV. However, considering that no evolutionary pressure acted on interactions between human and porcine molecules, the lack of redundancy across the species barrier (i.e. pULBP1 being the only ligand for human NKG2D) is not very surprising and in line with other known molecular incompatibilities between man and pig. In this regard, the aa identity between human ULBPs and pULBP1 is only around 35-52%. Remarkably, the level of conservation at the positions predicted to bind human NKG2D is relatively high. On the other hand, pig NKG2D has 67% aa identity with human NKG2D (236).

The non-classical human MHC molecule HLA-E is a potent inhibitory ligand for CD94/NKG2A receptor-bearing NK cells and It is generally believed that HLA-E, unlike classical MHC molecules, is not polymorphic and induces allogeneic T cell responses to a much lower degree (213). Therefore, transgeneic expression of HLA-E on pig organs has the potential for substantially alleviating human NK cell-mediated rejection of porcine xenografts. However, the stable expression of HLA-E on the cell-surface of pEC was previously shown to be problematic but was solvable by providing all three elements of the cell-surface HLA-E, *i.e.* the heavy chain, huβ2m, and a peptide, in a single peptide chain. An HLA-E SCT with VMAPRTLIL as peptide antigen (identical to the HLA-Cw*03 and UL40 signal sequence-derived peptides) folded correctly as evident by (i) its ability to bind several conformation-dependent mAbs, and (ii) the fact that HLA-E SCT functionally mirrored natural trimeric HLA-E in its ability to inhibit NK cell-mediated cytotoxicity and IFN-γ secretion (237) (see also results section 5.4.). HLA-E SCT may be further optimized by

changing the peptide antigen bound or by altering the HLA-E heavy chain. With respect to the peptide antigen, HLA-E bound with the HLA-G signal sequence-derived peptide, VMAPRTLFL, has an approximately 3-fold higher affinity for CD94/NKG2A than HLA-E bound with VMAPRTLIL (206). Furthermore, the use of a different HLA-E allele (E*0101), termed HLA-E^{G}, that exhibits higher affinity for most, if not all, peptides and has higher thermal stability (238), would further improve the HLA-E heavy chain component. This allele was chosen, in combination with the HLA-B7 signal sequence-derived peptide VMAPRTVLL, for the HLA-E^{sigB7} construct, used to generate HLA-E/huβ2m-transgenic pigs (see results section 5.5.).

There may be several explanations for the only partial protection achieved by the transgenic expression of HLA-E on pig cells. Primary human NK cells express a diverse repertoire of cell surface molecules potentially interacting with porcine target cells. The consequences might be either the lack of an inhibitory signal mediated by HLA-E SCT, or strong activating signals delivered to subsets of the polyclonal NK population. Diversity of the NK cell receptor repertoire is thought to be evolutionarily adaptive by not allowing a pathogen to completely shut off NK-mediated responses by interfering with one common molecular pathway. Moreover, it is well known that the polymorphism of MHC molecules in the population leads to different individual responses to the same pathogens (205). A clear correlation between CD94/NKG2A expression on NK clones and their cytotoxic potential against HLA-E-positive pEC was also shown earlier (110). Therefore, the lack of complete protection from polyclonal NK populations is most likely explained by the presence of subpopulations that were low or negative for CD94/NKG2A. The NK effector cells used *in vitro* were highly activated by stimulation with IL-2. It may be assumed that inhibitory HLA-E-specific signals are not sufficiently strong to override triggering signals delivered by activating receptors in the CD94/NKG2A dim NK cell subset. Supporting this notion, a weak allogeneic NK-mediated cytotoxicity (10-20% specific lysis) was observed using primary human target cells which presumably expressed normal levels of HLA-E molecules (own unpublished observations). In addition, freshly isolated, naïve human NK cells were almost completely inhibited by the expression of HLA-E on pEC (see results section 5.5.). Finally, it is well documented that the ligands for the activating receptor NKG2D are stress-inducible (150), a state that might be induced

during cell culture and does not necessarily need to correlate with the pEC phenotype of transplanted HLA-E transgenic pig organs *in vivo*.

In addition to being expressed on NK cells, the CD94/NKG2A inhibitory receptor is present on a subset of $CD8^+$ T cells that have undergone antigen-driven clonal expansion. Increased expression of CD94/NKG2A on human $CD8^+$ T cells also occurs following xenogeneic coculture with pEC (M. Crew, unpublished observations). Thus, HLA-E SCT may also be useful in reducing T cell responses to xenografts.

In summary, the clinical application of xenotransplantation on a routine basis may become reality one day. Nevertheless, it is important to combine several strategies to overcome the divergent xenogeneic immune responses. The generation of αGAL knockout pigs as well as pigs transgenic for human complement regulatory proteins, such as hDAF, to overcome hyperacute rejection, represented a great advance for the xenotransplantation research community. However, the identification and subsequent knocking out of the predominant ligands for non-αGAL xenoreactive Nab, may be needed to completely surmount HAR. Furthermore, xenograft survival would most likely benefit from knocking out the ligands for the human NK activating receptors, *i.e*. pULBP1 and the yet unidentified porcine ligands for human NKp44. In conclusion, for successful clinical xenotransplantation a crossbreeding of HLA-E/huβ2m-transgenic pigs with other transgenic and knockout pigs may be required to generate pigs resistant to the majority of rejection mechanisms as an appropriate organ source.

7. Appendix

7.1. References

1. Deschamps, J. Y., F. A. Roux, P. Sai, and E. Gouin. 2005. History of xenotransplantation. *Xenotransplantation.* 12:91-109.
2. Reemtsma, K., B. H. McCracken, J. Y. Schlegel, and M. A. Pearl. 1964. Heterotransplantation of the kidney: two clinical experiences. *Science* 143:700-702.
3. Starzl, T. E., T. L. Marchioro, G. N. Peters, C. H. Kirkpatrick, W. E. C. Wilson, K. E. Porter, D. Rifkind, D. A. Ogden, C. R. Hitchcock, and W. R. Waddell. 1964. Renal heterotransplantation from baboon to man: experience with six cases. *Transplantation* 2:752-776.
4. Cooper, D. K., B. Gollackner, and D. H. Sachs. 2002. Will the pig solve the transplantation backlog? *Annu. Rev. Med.* 53:133-147.
5. Dorling, A., K. Riesbeck, A. Warrens, and R. Lechler. 1997. Clinical xenotransplantation of solid organs. *Lancet* 349:867-871.
6. Cascalho, M. and J. L. Platt. 2001. The immunological barrier to xenotransplantation. *Immunity* 14:437-446.
7. Auchincloss, H., Jr. and D. H. Sachs. 1998. Xenogeneic transplantation. *Annu. Rev. Immunol.* 16:433-470.
8. Valdes-Gonzalez, R. A., L. M. Dorantes, G. N. Garibay, E. Bracho-Blanchet, A. J. Mendez, R. Davila-Perez, R. B. Elliott, L. Teran, and D. J. White. 2005. Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. *Eur. J. Endocrinol.* 153:419-427.
9. Platt, J. L., R. J. Fischel, A. J. Matas, S. A. Reif, R. M. Bolman, and F. H. Bach. 1991. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation* 52:214-220.
10. Bach, F. H., H. Winkler, C. Ferran, W. W. Hancock, and S. C. Robson. 1996. Delayed xenograft rejection. *Immunol. Today* 17:379-384.
11. Yamada, K., D. H. Sachs, and H. DerSimonian. 1995. The human anti-porcine xenogeneic T-cell response: Evidence for allelic specificity of MLR and for both direct and indirect pathways of recognition. *J. Immunol.* 155:5249-5256.
12. Calne, R. Y. 1970. Organ transplantation between widely disparate species. *Transplant. Proc.* 2:550-556.
13. Platt, J. L., B. J. Lindman, R. L. Geller, H. J. Noreen, J. L. Swanson, A. P. Dalmasso, and F. H. Bach. 1991. The role of natural antibodies in the activation of xenogenic endothelial cells. *Transplantation.* 52:1037-1043.
14. Dalmasso, A. P., G. M. Vercellotti, J. L. Platt, and F. H. Bach. 1991. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. Potential for prevention of xenograft hyperacute rejection. *Transplantation* 52:530-533.
15. Cooper, D. K., A. H. Good, E. Koren, R. Oriol, A. J. Malcolm, R. M. Ippolito, F. A. Neethling, Y. Ye, E. Romano, and N. Zuhdi. 1993. Identification of alpha-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. *Transpl. Immunol.* 1:198-205.
16. Galili, U., R. E. Mandrell, R. M. Hamadeh, S. B. Shohet, and J. M. Griffiss. 1988. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect. Immun.* 56:1730-1737.
17. Taniguchi, S., F. A. Neethling, E. Y. Korchagina, N. Bovin, Y. Ye, T. Kobayashi, M. Niekrasz, S. Li, E. Koren, R. Oriol, and D. K. C. Cooper. 1996. In vivo immunoadsorption of antipig antibodies in baboons using a specific Galα1-3Gal column. *Transplantation* 62:1379-1384.
18. Bach, F. H., S. C. Robson, H. Winkler, C. Ferran, K. M. Stuhlmeier, C. J. Wrighton, and W. W. Hancock. 1995. Barriers to xenotransplantation. *Nat. Med.* 1:869-873.
19. Platt, J. L., G. M. Vercellotti, B. J. Lindman, T. R. Oegema, Jr., F. H. Bach, and A. P. Dalmasso. 1990. Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection. *J. Exp. Med.* 171:1363-1368.
20. Leventhal, J. R., A. J. Matas, L. H. Sun, S. Reif, R. M. 3. Bolman, A. P. Dalmasso, and J. L. Platt. 1993. The immunopathology of cardiac xenograft rejection in the guinea pig-to-rat model. *Transplantation* 56:1-8.
21. Vercellotti, G. M., J. L. Platt, F. H. Bach, and A. P. Dalmasso. 1991. Neutrophil adhesion to xenogeneic endothelium via iC3b. *J. Immunol.* 146:730-734.

22. al Mohanna, F., K. Collison, R. Parhar, A. Kwaasi, B. Meyer, S. Saleh, S. Allen, S. al Sedairy, D. Stern, and M. Yacoub. 1997. Activation of naive xenogeneic but not allogeneic endothelial cells by human naive neutrophils: a potential occult barrier to xenotransplantation. *Am. J. Pathol.* 151:111-120.
23. Platt, J. L., R. J. Fischel, A. J. Matas, S. A. Reif, R. M. Bolman, and F. H. Bach. 1991. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation* 52:214-220.
24. Dorling, A., C. Stocker, T. Tsao, D. O. Haskard, and R. I. Lechler. 1996. In vitro accommodation of immortalized porcine endothelial cells: resistance to complement mediated lysis and down-regulation of VCAM expression induced by low concentrations of polyclonal human IgG antipig antibodies. *Transplantation* 62:1127-1136.
25. Millan, M. T., C. Geczy, K. M. Stuhlmeier, D. J. Goodman, C. Ferran, and F. H. Bach. 1997. Human monocytes activate porcine endothelial cells, resulting in increased E-selectin, interleukin-8, monocyte chemotactic protein-1, and plasminogen activator inhibitor-type-1 expression. *Transplantation* 63:421-429.
26. Robson, S. C., E. Kaczmarek, J. B. Siegel, D. Candinas, K. Koziak, M. Millan, W. W. Hancock, and F. H. Bach. 1997. Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J. Exp. Med.* 185:153-163.
27. Murray, A. G., M. M. Khodadoust, J. S. Pober, and A. L. Bothwell. 1994. Porcine aortic endothelial cells activate human T cells: direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD28. *Immunity* 1:57-63.
28. Choo, J. K., J. D. Seebach, V. Nickeleit, A. Shimizu, H. Lei, D. H. Sachs, and J. C. Madsen. 1997. Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium: implications for cell-mediated xenoreactivity. *Transplantation* 64:1315-1322.
29. Simon, A. R., A. N. Warrens, and M. Sykes. 1999. Efficacy of adhesive interactions in pig-to-human xenotransplantation. *Immunol. Today* 20:323-330.
30. Saadi, S. and J. L. Platt. 1998. Immunology of xenotransplantation. *Life Sci.* 62:365-387.
31. Maher, S. E., K. Karmann, W. Min, C. C. W. Hughes, J. S. Pober, and A. L. M. Bothwell. 1996. Porcine endothelial CD86 is a major costimulator of xenogeneic human T cells - Cloning, sequencing, and functional expression in human endothelial cells. *J. Immunol.* 157:3838-3844.
32. Davis, T. A., N. Craighead, A. J. Williams, A. Scadron, C. H. June, and K. P. Lee. 1996. Primary porcine endothelial cells express membrane-bound B7-2 (CD86) and a soluble factor that co-stimulate cyclosporin A- resistant and CD28-dependent human T cell proliferation. *Int. Immunol.* 8:1099-1111.
33. Grey, S. T. and W. W. Hancock. 1996. A physiologic anti-inflammatory pathway based on thrombomodulin expression and generation of activated protein C by human mononuclear phagocytes. *J. Immunol.* 156:2256-2263.
34. Malyguine, A. M., S. Saadi, R. A. Holzknecht, C. P. Patte, N. Sud, J. L. Platt, and J. R. Dawson. 1997. Induction of procoagulant function in porcine endothelial cells by human natural killer cells. *J. Immunol.* 159:4659-4664.
35. Sheikh, S., R. Parhar, A. Kwaasi, K. Collison, M. Yacoub, D. Stern, and F. al Mohanna. 2000. Alpha-gal-independent dual recognition and activation of xenogeneic endothelial cells and human naive natural killer cells. *Transplantation* 70:917-928.
36. Kwiatkowski, P., J. H. Artrip, N. M. Edwards, K. Lietz, S. Tugulea, R. E. Michler, I. F. McKenzie, M. S. Sandrin, and S. Itescu. 1999. High-level porcine endothelial cell expression of alpha(1,2)-fucosyltransferase reduces human monocyte adhesion and activation. *Transplantation* 67:219-226.
37. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301-314.
38. Zlotnik, A. and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity.* 12:121-127.
39. Schneider, M. K., P. Forte, and J. D. Seebach. 2001. Adhesive interactions between human NK cells and porcine endothelial cells. *Scand. J. Immunol.* 54:70-75.
40. Muller, W. A. 1995. The role of PECAM-1 (CD31) in leukocyte emigration: studies in vitro and in vivo. *J. Leukoc. Biol.* 57:523-528.
41. Piali, L., P. Hammel, C. Uherek, F. Bachmann, R. H. Gisler, D. Dunon, and B. A. Imhof. 1995. CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. *J. Cell Biol.* 130:451-460.

42. Miyagawa, S., R. Nakai, M. Yamada, M. Tanemura, Y. Ikeda, N. Taniguchi, and R. Shirakura. 1999. Regulation of natural killer cell-mediated swine endothelial cell lysis through genetic remodeling of a glycoantigen. *J. Biochem.* 126:1067-1073.
43. Inverardi, L., B. Clissi, A. L. Stolzer, J. R. Bender, M. S. Sandrin, and R. Pardi. 1997. Human natural killer lymphocytes directly recognize evolutionarily conserved oligosaccharide ligands expressed by xenogeneic tissues. *Transplantation* 63:1318-1330.
44. Baumann, B. C., M. K. Schneider, B. G. Lilienfeld, M. A. Antsiferova, D. M. Rhyner, R. J. Hawley, and J. D. Seebach. 2005. Endothelial cells derived from pigs lacking Gala(1,3)Gal: no reduction of human leukocyte adhesion and NK cytotoxicity. *Transplantation* 79:1067-1072.
45. Robinson, L. A., L. Tu, D. A. Steeber, O. Preis, J. L. Platt, and T. F. Tedder. 1998. The role of adhesion molecules in human leukocyte attachment to porcine vascular endothelium: implications for xenotransplantation. *J. Immunol.* 161:6931-6938.
46. Seebach, J. D. and G. L. Waneck. 1997. Natural killer cells in xenotransplantation. *Xenotransplantation* 4:201-211.
47. Kitchens, W. H., S. Uehara, C. M. Chase, R. B. Colvin, P. S. Russell, and J. C. Madsen. 2006. The changing role of natural killer cells in solid organ rejection and tolerance. *Transplantation.* 81:811-817.
48. Goodman, D. J., M. Von Albertini, A. Willson, M. T. Millan, and F. H. Bach. 1996. Direct activation of porcine endothelial cells by human natural killer cells. *Transplantation* 61:763-771.
49. Chan, D. V. and H. Auchincloss, Jr. 1996. Human anti-pig cell-mediated cytotoxicity in vitro involves non-T as well as T cell components. *Xenotransplantation* 3:158-165.
50. Itescu, S., P. Kwiatkowski, S. F. Wang, T. Blood, O. P. Minanov, S. Rose, and R. E. Michler. 1996. Circulating human mononuclear cells exhibit augmented lysis of pig endothelium after activation with interleukin 2. *Transplantation* 62:1927-1933.
51. Malyguine, A. M., S. Saadi, J. L. Platt, and J. R. Dawson. 1996. Human natural killer cells induce morphologic changes in porcine endothelial cell monolayers. *Transplantation* 61:161-164.
52. Donnelly, C. E., C. Yatko, E. W. Johnson, and A. S. Edge. 1997. Human natural killer cells account for non-MHC class I- restricted cytolysis of porcine cells. *Cell Immunol.* 175:171-178.
53. Yi, S., X. Feng, W. J. Hawthorne, A. T. Patel, S. N. Walters, and P. J. O'Connell. 2002. CD4+ T cells initiate pancreatic islet xenograft rejection via an interferon-gamma-dependent recruitment of macrophages and natural killer cells. *Transplantation* 73:437-446.
54. Rieben, R. and J. D. Seebach. 2005. Xenograft rejection: IgG(1), complement and NK cells team up to activate and destroy the endothelium. *Trends Immunol.* 26:2-5.
55. Schaapherder, A. F., M. R. Daha, M. T. te Bulte, F. J. Van der Woude, and H. G. Gooszen. 1994. Antibody-dependent cell-mediated cytotoxicity against porcine endothelium induced by a majority of human sera. *Transplantation* 57:1376-1382.
56. Yin, D., H. Zeng, L. Ma, J. Shen, H. Xu, G. W. Byrne, and A. S. Chong. 2004. Cutting Edge: NK cells mediate IgG1-dependent hyperacute rejection of xenografts. *J. Immunol.* 172:7235-7238.
57. Khalfoun, B., D. Barrat, H. Watier, M. C. Machet, B. Arbeille-Brassart, J. G. Riess, H. Salmon, Y. Gruel, P. Bardos, and Y. Lebranchu. 2000. Development of an ex vivo model of pig kidney perfused with human lymphocytes. Analysis of xenogeneic cellular reactions. *Surgery* 128:447-457.
58. Kirk, A. D., J. S. Heinle, J. R. Mault, and F. Sanfilippo. 1993. Ex vivo characterization of human anti-porcine hyperacute cardiac rejection. *Transplantation* 56:785-793.
59. Inverardi, L. and R. Pardi. 1994. Early events in cell-mediated recognition of vascularized xenografts: cooperative interactions between selected lymphocyte subsets and natural antibodies. *Immunol. Rev.* 141:71-93.
60. Itescu, S., P. Kwiatkowski, J. H. Artrip, S. F. Wang, J. Ankersmit, O. P. Minanov, and R. E. Michler. 1998. Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. *Hum. Immunol.* 59:275-286.
61. Lin, Y., M. Vandeputte, and M. Waer. 1997. Natural killer cell- and macrophage-mediated rejection of concordant xenografts in the absence of T and B cell responses. *J. Immunol.* 158:5658-5667.
62. Xia, G., P. Ji, O. Rutgeerts, and M. Waer. 2000. Natural killer cell- and macrophage mediated discordant guinea pig-->rat xenograft rejection in the absence of complement, xenoantibody and T cell immunity. *Transplantation* 70:86-93.

63. Lin, Y., J. Goebels, G. Xia, P. Ji, M. Vandeputte, and M. Waer. 1998. Induction of specific transplantation tolerance across xenogeneic barriers in the T-independent immune compartment. *Nature Med.* 4:173-180.
64. Umesue, M., H. Mayumi, Y. Nishimura, Y. Y. Kong, K. Omoto, Y. Murakami, and K. Nomoto. 1996. Donor-specific prolongation of rat skin graft survival induced by rat-donor cells and cyclophosphamide under coadministration of monoclonal antibodies against T cell receptor alpha beta and natural killer cells in mice. *Transplantation.* 61:116-124.
65. Korsgren, O. 1997. Acute cellular xenograft rejection. *Xenotransplantation* 4:11-19.
66. Karlsson-Parra, A., A. Ridderstad, A. C. Wallgren, E. Moller, H. G. Ljunggren, and O. Korsgren. 1996. Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice. *Transplantation* 61:1313-1320.
67. Sharabi, Y., I. Aksentijevich, T. M. Sundt III, D. H. Sachs, and M. Sykes. 1990. Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. *J. Exp. Med.* 172:195-202.
68. Birmele, B., G. Thibault, H. Watier, I. Vallee, Y. Gruel, H. Nivet, H. Salmon, P. Bardos, and Y. Lebranchu. 1994. Human peripheral blood lymphocyte adhesion to xenogeneic porcine aortic endothelial cells: preferential adhesion of CD3-CD16+ NK cells. *Transplant. Proc.* 26:1150-1151.
69. Birmele, B., G. Thibault, H. Nivet, Y. Gruel, P. Bardos, and Y. Lebranchu. 1996. Human lymphocyte adhesion to xenogeneic porcine endothelial cells: modulation by human TNF-alpha and involvement of VLA-4 and LFA-1. *Transpl. Immunol.* 4:265-270.
70. Schneider, M. K., M. Strasser, U. O. Gilli, M. Kocher, R. Moser, and J. D. Seebach. 2002. Rolling adhesion of human NK cells to porcine endothelial cells mainly relies on CD49d-CD106 interactions. *Transplantation* 73:789-796.
71. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187-376.
72. Sullivan, J. A., H. F. Oettinger, D. H. Sachs, and A. S. Edge. 1997. Analysis of polymorphism in porcine MHC class I genes: alterations in signals recognized by human cytotoxic lymphocytes. *J. Immunol.* 159:2318-2326.
73. Forte, P., B. G. Lilienfeld, B. C. Baumann, and J. D. Seebach. 2005. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D. *J. Immunol.* (in press)
74. Seebach, J. D., C. Comrack, S. Germana, C. LeGuern, D. H. Sachs, and H. DerSimonian. 1997. HLA-Cw3 expression on porcine endothelial cells protects against xenogeneic cytotoxicity mediated by a subset of human NK cells. *J. Immunol.* 159:3655-3661.
75. Forte, P., L. Pazmany, U. B. Matter-Reissmann, G. Stussi, M. K. Schneider, and J. D. Seebach. 2001. HLA-G Inhibits Rolling Adhesion of Activated Human NK Cells on Porcine Endothelial Cells. *J. Immunol.* 167:6002-6008.
76. Sharland, A., J. H. Lee, S. Saidman, and G. L. Waneck. 2003. CD8-interaction mutant HLA-Cw3 molecules protect porcine cells from human natural killer cell-mediated antibody-dependent cellular cytotoxicity without stimulating cytotoxic T lymphocytes. *Transplantation* 76:1615-1622.
77. Sasaki, H., X. C. Xu, and T. Mohanakumar. 1999. HLA-E and HLA-G expression on porcine endothelial cells inhibit xenoreactive human NK cells through CD94/NKG2-dependent and -independent pathways. *J. Immunol.* 163:6301-6305.
78. Matsunami, K., S. Miyagawa, R. Nakai, M. Yamada, and R. Shirakura. 2002. Modulation of the leader peptide sequence of the HLA-E gene up-regulates its expression and down-regulates natural killer cell-mediated swine endothelial cell lysis. *Transplantation* 73:1582-1589.
79. Sharland, A., A. Patel, J. H. Lee, A. E. Cestra, S. Saidman, and G. L. Waneck. 2002. Genetically modified HLA class I molecules able to inhibit human NK cells without provoking alloreactive CD8+ CTLs. *J. Immunol.* 168:3266-3274.
80. Seebach, J. D., L. Pazmany, G. L. Waneck, F. Minja, S. Germana, C. LeGuern, and D. H. Sachs. 1999. HLA-G expression on porcine endothelial cells protects partially against direct human NK cytotoxicity but not against ADCC. *Transplant. Proc.* 31:1864-1865.
81. Forte, P., B. G. Lilienfeld, B. C. Baumann, and J. D. Seebach. 2005. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D. *J. Immunol.* 175:5463-5470.
82. Gritsch, H. A., R. M. Glaser, D. W. Emery, L. A. Lee, C. V. Smith, T. Sablinski, J. S. Arn, D. H. Sachs, and M. Sykes. 1994. The importance of nonimmune factors in reconstitution by discordant xenogeneic hematopoietic cells. *Transplantation.* 57:906-917.
83. Yang, Y.-G., J. Sergio, K. Swenson, R. M. Glaser, R. Monroy, and M. Sykes. 1996. Donor-specific growth factors promotes swine hematopoiesis in severe combined immune deficient mice. *Xenotransplantation* 3:92-101.

84. Gritsch, H. A. and M. Sykes. 1996. Host marrow has a competitive advantage that limits its donor hematopoietic repopulation in mixed xenogeneic chimeras. *Xenotransplantation* 3:312-320.
85. Fishman, J. A. and C. Patience. 2004. Xenotransplantation: infectious risk revisited. *Am. J. Transplant.* 4:1383-1390.
86. Patience, C., W. M. Switzer, Y. Takeuchi, D. J. Griffiths, M. E. Goward, W. Heneine, J. P. Stoye, and R. A. Weiss. 2001. Multiple groups of novel retroviral genomes in pigs and related species. *J. Virol.* 75:2771-2775.
87. Klymiuk, N., M. Muller, G. Brem, and B. Aigner. 2002. Characterization of porcine endogenous retrovirus gamma pro-pol nucleotide sequences. *J. Virol.* 76:11738-11743.
88. Oldmixon, B. A., J. C. Wood, T. A. Ericsson, C. A. Wilson, M. E. White-Scharf, G. Andersson, J. L. Greenstein, H. J. Schuurman, and C. Patience. 2002. Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. *J. Virol.* 76:3045-3048.
89. Ericsson, T. A., Y. Takeuchi, C. Templin, G. Quinn, S. F. Farhadian, J. C. Wood, B. A. Oldmixon, K. M. Suling, J. K. Ishii, Y. Kitagawa, T. Miyazawa, D. R. Salomon, R. A. Weiss, and C. Patience. 2003. Identification of receptors for pig endogenous retrovirus. *Proc. Natl. Acad. Sci. U. S. A.* 100:6759-6764.
90. Takeuchi, Y., C. Patience, S. Magre, R. A. Weiss, P. T. Banerjee, P. Le Tissier, and J. P. Stoye. 1998. Host range and interference studies of three classes of pig endogenous retrovirus. *J. Virol.* 72:9986-9991.
91. Akiyoshi, D. E., M. Denaro, H. Zhu, J. L. Greenstein, P. Banerjee, and J. A. Fishman. 1998. Identification of a full-length cDNA for an endogenous retrovirus of miniature swine. *J. Virol.* 72:4503-4507.
92. Le Tissier, P., J. P. Stoye, Y. Takeuchi, C. Patience, and R. A. Weiss. 1997. Two sets of human-tropic pig retrovirus. *Nature.* 389:681-682.
93. Patience, C., G. S. Patton, Y. Takeuchi, R. A. Weiss, M. O. McClure, L. Rydberg, and M. E. Breimer. 1998. No evidence of pig DNA or retroviral infection in patients with short-term extracorporeal connection to pig kidneys. *Lancet* 352:699-701.
94. Paradis, K., G. Langford, Z. Long, W. Heneine, P. Sandstrom, W. M. Switzer, L. E. Chapman, C. Lockey, D. Onions, and E. Otto. 1999. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science* 285:1236-1241.
95. Cunningham, D. A., C. Herring, X. M. Fernandez-Suarez, A. J. Whittam, K. Paradis, and G. A. Langford. 2001. Analysis of patients treated with living pig tissue for evidence of infection by porcine endogenous retroviruses. *Trends Cardiovasc. Med.* 11:190-196.
96. Wilhelm, M., J. A. Fishman, R. Pontikis, A. M. Aubertin, and F. X. Wilhelm. 2002. Susceptibility of recombinant porcine endogenous retrovirus reverse transcriptase to nucleoside and non-nucleoside inhibitors. *Cell Mol. Life Sci.* 59:2184-2190.
97. Fishman, J. A. and R. H. Rubin. 1998. Infection in organ-transplant recipients. *N. Engl. J. Med.* 338:1741-1751.
98. Leventhal, J. R., H. C. Flores, S. A. Gruber, J. Figueroa, J. L. Platt, J. C. Manivel, F. H. Bach, A. J. Matas, and R. M. Bolman. 1992. Evidence that 15-deoxyspergualin inhibits natural antibody production but fails to prevent hyperacute rejection in a discordant xenograft model. *Transplantation* 54:26-31.
99. Pruitt, S. K., R. R. Bollinger, B. H. Collins, J. Marsh-HC, J. L. Levin, A. R. Rudolph, W. M. Baldwin, III, and F. Sanfilippo. 1997. Effect of continuous complement inhibition using soluble complement receptor type 1 on survival of pig-to-primate cardiac xenografts. *Transplantation* 63:900-902.
100. Rollins, S. A., L. A. Matis, J. P. Springhorn, E. Setter, and D. W. Wolff. 1995. Monoclonal antibodies directed against human C5 and C8 block complement-mediated damage of xenogeneic cells and organs. *Transplantation* 60:1284-1292.
101. Phelps, C. J., C. Koike, T. D. Vaught, J. Boone, K. D. Wells, S. H. Chen, S. Ball, S. M. Specht, I. A. Polejaeva, J. A. Monahan, P. M. Jobst, S. B. Sharma, A. E. Lamborn, A. S. Garst, M. Moore, A. J. Demetris, W. A. Rudert, R. Bottino, S. Bertera, M. Trucco, T. E. Starzl, Y. Dai, and D. L. Ayares. 2003. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 299:411-414.
102. Kolber-Simonds, D., L. Lai, S. R. Watt, M. Denaro, S. Arn, M. L. Augenstein, J. Betthauser, D. B. Carter, J. L. Greenstein, Y. Hao, G. S. Im, Z. Liu, G. D. Mell, C. N. Murphy, K. W. Park, A. Rieke, D. J. Ryan, D. H. Sachs, E. J. Forsberg, R. S. Prather, and R. J. Hawley. 2004. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc. Natl. Acad. Sci. U. S. A* 101:7335-7340.

103. Kuwaki, K., Y. L. Tseng, F. J. Dor, A. Shimizu, S. L. Houser, T. M. Sanderson, C. J. Lancos, D. D. Prabharasuth, J. Cheng, K. Moran, Y. Hisashi, N. Mueller, K. Yamada, J. L. Greenstein, R. J. Hawley, C. Patience, M. Awwad, J. A. Fishman, S. C. Robson, H. J. Schuurman, D. H. Sachs, and D. K. Cooper. 2005. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat. Med.* 11:29-31.
104. Yamada, K., K. Yazawa, A. Shimizu, T. Iwanaga, Y. Hisashi, M. Nuhn, P. O'malley, S. Nobori, P. A. Vagefi, C. Patience, J. Fishman, D. K. Cooper, R. J. Hawley, J. Greenstein, H. J. Schuurman, M. Awwad, M. Sykes, and D. H. Sachs. 2005. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat. Med.* 11:32-34.
105. Tseng, Y. L., K. Kuwaki, F. J. Dor, A. Shimizu, S. Houser, Y. Hisashi, K. Yamada, S. C. Robson, M. Awwad, H. J. Schuurman, D. H. Sachs, and D. K. Cooper. 2005. alpha1,3-Galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching 6 months. *Transplantation.* 80:1493-1500.
106. Adams, D. H., A. Kadner, R. H. Chen, and R. S. Farivar. 2001. Human membrane cofactor protein (MCP, CD 46) protects transgenic pig hearts from hyperacute rejection in primates. *Xenotransplantation.* 8:36-40.
107. Fodor, W. L., B. L. Williams, L. A. Matis, J. A. Madri, S. A. Rollins, J. W. Knight, W. Velander, and S. P. Squinto. 1994. Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. *Proc. Natl. Acad. Sci. U. S. A.* 91:11153-11157.
108. Byrne, G. W., K. R. McCurry, M. J. Martin, S. M. McClellan, J. L. Platt, and J. S. Logan. 1997. Transgenic pigs expressing human CD59 and decay-accelerating factor produce an intrinsic barrier to complement-mediated damage. *Transplantation* 63:149-155.
109. Tran, T. H., S. Grey, J. Anrather, F. Steinhauslin, F. H. Bach, and H. Winkler. 1998. Regulated and endothelial cell-specific expression of Fas ligand: an in vitro model for a strategy aiming at inhibiting xenograft rejection. *Transplantation* 66:1126-1131.
110. Forte, P., B. C. Baumann, E. H. Weiss, and J. D. Seebach. 2005. HLA-E expression on porcine cells: protection from human NK cytotoxicity depends on peptide loading. *Am. J. Transplant.* 5:2085-2093.
111. Nikolic, B. and M. Sykes. 1997. Bone marrow chimerism and transplantation tolerance. *Curr. Opin. Immunol.* 9:634-640.
112. Nikolic, B., J. P. Gardner, D. T. Scadden, J. S. Arn, D. H. Sachs, and M. Sykes. 1999. Normal development in porcine thymus grafts and specific tolerance of human T cells to porcine donor MHC. *J. Immunol.* 162:3402-3407.
113. Yokoyama, W. M., S. Kim, and A. R. French. 2004. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* 22:405-429.
114. French, A. R. and W. M. Yokoyama. 2003. Natural killer cells and viral infections. *Curr. Opin. Immunol.* 15:45-51.
115. Cooper, M. A., T. A. Fehniger, and M. A. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol.* 22:633-640.
116. Andre, P., R. Biassoni, M. Colonna, D. Cosman, L. L. Lanier, E. O. Long, M. Lopez-Botet, A. Moretta, L. Moretta, P. Parham, J. Trowsdale, E. Vivier, N. Wagtmann, and M. J. Wilson. 2001. New nomenclature for MHC receptors. *Nat. Immunol.* 2:661.
117. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319:675-678.
118. Ljunggren, H. G. and K. Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 11:237-244.
119. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19:197-223.:197-223.
120. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* 3:781-790.
121. Ravetch, J. V. and L. L. Lanier. 2000. Immune inhibitory receptors. *Science.* 290:84-89.
122. Muta, T., T. Kurosaki, Z. Misulovin, M. Sanchez, M. C. Nussenzweig, and J. V. Ravetch. 1994. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. *Nature.* 369:340.
123. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23:225-74.:225-274.
124. Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358:66-70.

125. Smith, K. M., J. Wu, A. B. Bakker, J. H. Phillips, and L. L. Lanier. 1998. Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J. Immunol.* 161:7-10.
126. Natarajan, K., N. Dimasi, J. Wang, R. A. Mariuzza, and D. H. Margulies. 2002. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu. Rev. Immunol.* 20:853-885.
127. Moretta, A., C. Bottino, D. Pende, G. Tripodi, G. Tambussi, O. Viale, A. Orengo, M. Barbaresi, A. Merli, and E. Ciccone. 1990. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J. Exp. Med.* 172:1589-1598.
128. Litwin, V., J. Gumperz, P. Parham, J. H. Phillips, and L. L. Lanier. 1994. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J. Exp. Med.* 180:537-543.
129. Phillips, J. H., J. E. Gumperz, P. Parham, and L. L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science* 268:403-405.
130. Colonna, M. and J. Samaridis. 1995. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells [see comments]. *Science* 268:405-408.
131. Malnati, M. S., M. Peruzzi, K. C. Parker, W. E. Biddison, E. Ciccone, A. Moretta, and E. O. Long. 1995. Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science* 267:1016-1018.
132. Houchins, J. P., T. Yabe, C. McSherry, and F. H. Bach. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J. Exp. Med.* 173:1017-1020.
133. Shum, B. P., L. R. Flodin, D. G. Muir, R. Rajalingam, S. I. Khakoo, S. Cleland, L. A. Guethlein, M. Uhrberg, and P. Parham. 2002. Conservation and variation in human and common chimpanzee CD94 and NKG2 genes. *J. Immunol.* 168:240-252.
134. Houchins, J. P., L. L. Lanier, E. C. Niemi, J. H. Phillips, and J. C. Ryan. 1997. Natural killer cell cytolytic activity is inhibited by NKG2- A and activated by NKG2-C. *J. Immunol.* 158:3603-3609.
135. Aramburu, J., M. A. Balboa, A. Ramirez, A. Silva, A. Acevedo, F. Sanchez-Madrid, M. O. de Landazuri, and M. Lopez-Botet. 1990. A novel functional cell surface dimer (Kp43) expressed by natural killer cells and T cell receptor-gamma/delta+ T lymphocytes. I. Inhibition of the IL-2-dependent proliferation by anti-Kp43 monoclonal antibody. *J. Immunol.* 144:3238-3247.
136. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187:813-818.
137. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. E. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. U. S. A* 95:5199-5204.
138. Wei, X. H. and H. T. Orr. 1990. Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. *Hum. Immunol.* 29:131-142.
139. Braud, V. M., D. S. Allan, D. Wilson, and A. J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* 8:1-10.
140. Forte, P., B. C. Baumann, E. H. Weiss, and J. D. Seebach. 2005. HLA-E Expression on Porcine Cells: Protection from Human NK Cytotoxicity Depends on Peptide Loading. *Am. J. Transplant.* (in press)
141. Crew, M. D., M. J. Cannon, B. Phanavanh, and C. N. Garcia-Borges. 2005. An HLA-E single chain trimer inhibits human NK cell reactivity towards porcine cells. *Mol. Immunol.* (in press)
142. Sivori, S., D. Pende, C. Bottino, E. Marcenaro, A. Pessino, R. Biassoni, L. Moretta, and A. Moretta. 1999. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur. J. Immunol.* 29:1656-1666.
143. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J. Exp. Med.* 187:2065-2072.

144. Vitale, M., M. Falco, R. Castriconi, S. Parolini, R. Zambello, G. Semenzato, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2001. Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur. J. Immunol.* 31:233-242.
145. Sivori, S., S. Parolini, M. Falco, E. Marcenaro, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2000. 2B4 functions as a co-receptor in human NK cell activation. *Eur. J. Immunol.* 30:787-793.
146. Bottino, C., M. Falco, S. Parolini, E. Marcenaro, R. Augugliaro, S. Sivori, E. Landi, R. Biassoni, L. D. Notarangelo, L. Moretta, and A. Moretta. 2001. NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J. Exp. Med.* 194:235-246.
147. Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. An activating immunoreceptor complex formed by NKG2D and DAP10 [see comments]. *Science* 285:730-732.
148. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285:727-729.
149. Chang, C., J. Dietrich, A. G. Harpur, J. A. Lindquist, A. Haude, Y. W. Loke, A. King, M. Colonna, J. Trowsdale, and M. J. Wilson. 1999. Cutting edge: KAP10, a novel transmembrane adapter protein genetically linked to DAP12 but with unique signaling properties. *J. Immunol.* 163:4651-4654.
150. Sutherland, C. L., N. J. Chalupny, and D. Cosman. 2001. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. *Immunol. Rev.* 181:185-192.
151. Collins, R. W. 2004. Human MHC class I chain related (MIC) genes: their biological function and relevance to disease and transplantation. *Eur. J. Immunogenet.* 31:105-114.
152. Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity.* 14:123-133.
153. Radaev, S., B. Rostro, A. G. Brooks, M. Colonna, and P. D. Sun. 2001. Conformational plasticity revealed by the cocrystal structure of NKG2D and its class I MHC-like ligand ULBP3. *Immunity.* 15:1039-1049.
154. Stephens, H. A. 2001. MICA and MICB genes: can the enigma of their polymorphism be resolved? *Trends Immunol.* 22:378-385.
155. Garcia-Borges, C. N., B. Phanavanh, S. Saraswati, R. A. Dennis, and M. D. Crew. 2005. Molecular cloning and characterization of a porcine UL16 binding protein (ULBP)-like cDNA. *Mol. Immunol.* 42:665-671.
156. Chardon, P., C. Rogel-Gaillard, L. Cattolico, S. Duprat, M. Vaiman, and C. Renard. 2001. Sequence of the swine major histocompatibility complex region containing all non-classical class I genes. *Tissue Antigens* 57:55-65.
157. Lilienfeld, B. G., C. Garcia-Borges, M. D. Crew, and J. D. Seebach. 2006. Porcine UL16-binding protein 1 expressed on the surface of endothelial cells triggers human NK cytotoxicity through NKG2D. *J. Immunol.* 177:2146-2152.
158. Ferrini, S., S. Miescher, M. R. Zocchi, F. von, V, and A. Moretta. 1987. Phenotypic and functional characterization of recombinant interleukin 2 (rIL 2)-induced activated killer cells: analysis at the population and clonal levels. *J. Immunol.* 138:1297-1302.
159. Cantoni, C., C. Bottino, M. Vitale, A. Pessino, R. Augugliaro, A. Malaspina, S. Parolini, L. Moretta, A. Moretta, and R. Biassoni. 1999. NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J. Exp. Med.* 189:787-796.
160. Porgador, A. 2005. Natural cytotoxicity receptors: pattern recognition and involvement of carbohydrates. *ScientificWorldJournal.* 5:151-4.:151-154.
161. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* 190:1505-1516.
162. Pessino, A., S. Sivori, C. Bottino, A. Malaspina, L. Morelli, L. Moretta, R. Biassoni, and A. Moretta. 1998. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J. Exp. Med.* 188:953-960.
163. Seebach, J. D., M. K. Schneider, C. A. Comrack, A. LeGuern, S. A. Kolb, P. A. Knolle, S. Germana, H. DerSimonian, C. LeGuern, and D. H. Sachs. 2001. Immortalized bone-marrow derived pig endothelial cells. *Xenotransplantation* 8:48-61.

164. Huang, C. A., Y. Fuchimoto, Z. L. Gleit, T. Ericsson, A. Griesemer, R. Scheier-Dolberg, E. Melendy, H. Kitamura, J. A. Fishman, J. A. Ferry, N. L. Harris, C. Patience, and D. H. Sachs. 2001. Posttransplantation lymphoproliferative disease in miniature swine after allogeneic hematopoietic cell transplantation: similarity to human PTLD and association with a porcine gammaherpesvirus. *Blood* 97:1467-1473.
165. Pende, D., L. Accame, L. Pareti, A. Mazzocchi, A. Moretta, G. Parmiani, and L. Moretta. 1998. The susceptibility to natural killer cell-mediated lysis of HLA class I-positive melanomas reflects the expression of insufficient amounts of different HLA class I alleles. *Eur. J. Immunol.* 28:2384-2394.
166. Seebach, J. D., K. Yamada, I. McMorrow, D. H. Sachs, and H. DerSimonian. 1996. Xenogeneic human anti-pig cytotoxicity mediated by activated natural killer cells. *Xenotransplantation* 3:188-197.
167. Kwiatkowski, P., J. H. Artrip, R. John, N. M. Edwards, S. F. Wang, R. E. Michler, and S. Itescu. 1999. Induction of swine major histocompatibility complex class I molecules on porcine endothelium by tumor necrosis factor-alpha reduces lysis by human natural killer cells. *Transplantation* 67:211-218.
168. Matter-Reissmann, U. B., P. Forte, M. K. Schneider, L. Filgueira, P. Groscurth, and J. D. Seebach. 2002. Xenogeneic human NK cytotoxicity against porcine endothelial cells is perforin/granzyme B dependent and not inhibited by Bcl-2 overexpression. *Xenotransplantation* 9:325-337.
169. Salcedo, T. W., L. Azzoni, S. F. Wolf, and B. Perussia. 1993. Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *J. Immunol.* 151:2511-2520.
170. Martin, A. M., J. K. Kulski, C. Witt, P. Pontarotti, and F. T. Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81-88.
171. Parham, P. 1997. Events in the adaptation of natural killer cell receptors to MHC class I polymorphisms. *Res. Immunol.* 148:190-194.
172. Volz, A., H. Wende, K. Laun, and A. Ziegler. 2001. Genesis of the ILT/LIR/MIR clusters within the human leukocyte receptor complex. *Immunol. Rev.* 181:39-51.
173. Barten, R., M. Torkar, A. Haude, J. Trowsdale, and M. J. Wilson. 2001. Divergent and convergent evolution of NK-cell receptors. *Trends Immunol.* 22:52-57.
174. Chen, F. X., J. Tang, N. L. Li, B. H. Shen, Y. Zhou, J. Xie, and K. Y. Chou. 2003. Novel SLA class I alleles of Chinese pig strains and their significance in xenotransplantation. *Cell Res.* 13:285-294.
175. Garcia-Borges, C. N., B. Phanavanh, S. Saraswati, R. A. Dennis, and M. D. Crew. 2004. Molecular cloning and characterization of a porcine UL16 binding protein (ULBP)-like cDNA. *Mol. Immunol.* (in press)
176. Renard, C., P. Chardon, and M. Vaiman. 2003. The phylogenetic history of the MHC class I gene families in pig, including a fossil gene predating mammalian radiation. *J. Mol. Evol.* 57:420-434.
177. Hankey, K. G., C. B. Drachenberg, J. C. Papadimitriou, D. K. Klassen, B. Philosophe, S. T. Bartlett, V. Groh, T. Spies, and D. L. Mann. 2002. MIC expression in renal and pancreatic allografts. *Transplantation* 73:304-306.
178. Zwirner, N. W., C. Y. Marcos, F. Mirbaha, Y. Zou, and P. Stastny. 2000. Identification of MICA as a new polymorphic alloantigen recognized by antibodies in sera of organ transplant recipients. *Hum. Immunol.* 61:917-924.
179. Baumann, B. C., P. Forte, R. J. Hawley, R. Rieben, M. K. Schneider, and J. D. Seebach. 2004. Lack of galactose-alpha-1,3-galactose expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity. *J. Immunol.* 172:6460-6467.
180. Chen, G., H. Qian, T. Starzl, H. Sun, B. Garcia, X. Wang, Y. Wise, Y. Liu, Y. Xiang, L. Copeman, W. Liu, A. Jevnikar, W. Wall, D. K. Cooper, N. Murase, Y. Dai, W. Wang, Y. Xiong, D. J. White, and R. Zhong. 2005. Acute rejection is associated with antibodies to non-Gal antigens in baboons using Gal-knockout pig kidneys. *Nat. Med.* 11:1295-1298.
181. Young, N. T. 2004. Immunobiology of natural killer lymphocytes in transplantation. *Transplantation.* 78:1-6.
182. Gonzalez, S., V. Groh, and T. Spies. 2006. Immunobiology of human NKG2D and its ligands. *Curr. Top. Microbiol. Immunol.* 298:121-38.:121-138.
183. Yim, D., H. B. Jie, J. Sotiriadis, Y. S. Kim, K. S. Kim, M. F. Rothschild, L. L. Lanier, and Y. B. Kim. 2001. Molecular cloning and characterization of pig immunoreceptor DAP10 and NKG2D. *Immunogenetics.* 53:243-249.

184. Matsunami, K., S. Miyagawa, R. Nakai, M. Yamada, and R. Shirakura. 2000. Protection against natural killer-mediated swine endothelial cell lysis by HLA-G and HLA-E. *Transplant. Proc.* 32:939-940.
185. Forte, P., U. B. Matter-Reissmann, M. Strasser, M. K. Schneider, and J. D. Seebach. 2000. Porcine aortic endothelial cells transfected with HLA-G are partially protected from xenogeneic human NK cytotoxicity. *Hum. Immunol.* 61:1066-1073.
186. Paddison, P. J., A. A. Caudy, E. Bernstein, G. J. Hannon, and D. S. Conklin. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16:948-958.
187. Jackson, A. L., S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, and P. S. Linsley. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21:635-637.
188. Cerwenka, A., A. B. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips, and L. L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity.* 12:721-727.
189. Seo, J. W., L. Walter, and E. Gunther. 2001. Genomic analysis of MIC genes in rhesus macaques. *Tissue Antigens* 58:159-165.
190. Yang, Y. G. 2004. Application of xenogeneic stem cells for induction of transplantation tolerance: present state and future directions. *Springer Semin. Immunopathol.* 26:187-200.
191. Fehr, T. and M. Sykes. 2004. Tolerance induction in clinical transplantation. *Transpl. Immunol.* 13:117-130.
192. Raulet, D. H. and R. E. Vance. 2006. Self-tolerance of natural killer cells. *Nat. Rev. Immunol.* 6:520-531.
193. Wu, J., N. J. Chalupny, T. J. Manley, S. R. Riddell, D. Cosman, and T. Spies. 2003. Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. *J. Immunol.* 170:4196-4200.
194. Welte, S. A., C. Sinzger, S. Z. Lutz, H. Singh-Jasuja, K. L. Sampaio, U. Eknigk, H. G. Rammensee, and A. Steinle. 2003. Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein. *Eur. J. Immunol.* 33:194-203.
195. Roost, H. P., M. F. Bachmann, A. Haag, U. Kalinke, V. Pliska, H. Hengartner, and R. M. Zinkernagel. 1995. Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc. Natl. Acad. Sci. U. S. A.* 92:1257-1261.
196. Gollackner, B., N. J. Mueller, S. Houser, I. Qawi, D. Soizic, C. Knosalla, L. Buhler, F. J. Dor, M. Awwad, D. H. Sachs, D. K. Cooper, S. C. Robson, and J. A. Fishman. 2003. Porcine cytomegalovirus and coagulopathy in pig-to-primate xenotransplantation. *Transplantation.* 75:1841-1847.
197. Mueller, N. J., R. N. Barth, S. Yamamoto, H. Kitamura, C. Patience, K. Yamada, D. K. Cooper, D. H. Sachs, A. Kaur, and J. A. Fishman. 2002. Activation of cytomegalovirus in pig-to-primate organ xenotransplantation. *J. Virol.* 76:4734-4740.
198. Batten, P., M. H. Yacoub, and M. L. Rose. 1996. Effect of human cytokines (IFN-gamma, TNF-alpha, IL-1 beta, IL- 4) on porcine endothelial cells: induction of MHC and adhesion molecules and functional significance of these changes. *Immunology* 87:127-133.
199. Maurus, C. F., M. K. Schneider, D. Schmidt, G. Zund, and J. D. Seebach. 2006. Activation of human microvascular endothelial cells with TNF-alpha and hypoxia/reoxygenation enhances NK-cell adhesion, but not NK-Cytotoxicity. *Transplantation.* 81:1204-1211.
200. Moretta, L. and A. Moretta. 2004. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* 23:255-259.
201. Crew, M. D., M. J. Cannon, B. Phanavanh, and C. N. Garcia-Borges. 2005. An HLA-E single chain trimer inhibits human NK cell reactivity towards porcine cells. *Mol. Immunol.* 42:1205-1214.
202. Burshtyn, D. N., J. Shin, C. Stebbins, and E. O. Long. 2000. Adhesion to target cells is disrupted by the killer cell inhibitory receptor. *Curr. Biol.* 10:777-780.
203. Carrillo, A., S. Chamorro, M. Rodriguez-Gago, B. Alvarez, M. J. Molina, J. I. Rodriguez-Barbosa, A. Sanchez, P. Ramirez, A. Munoz, J. Dominguez, P. Parrilla, and J. Yelamos. 2002. Isolation and characterization of immortalized porcine aortic endothelial cell lines. *Vet. Immunol. Immunopathol.* 89:91-98.
204. Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160:4951-4960.
205. Trowsdale, J. 2005. HLA genomics in the third millennium. *Curr. Opin. Immunol.* 17:498-504.

206. Vales-Gomez, M., H. T. Reyburn, R. A. Erskine, M. Lopez-Botet, and J. L. Strominger. 1999. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* 18:4250-4260.
207. Kuwaki, K., Y. L. Tseng, F. J. Dor, A. Shimizu, S. L. Houser, T. M. Sanderson, C. J. Lancos, D. D. Prabharasuth, J. Cheng, K. Moran, Y. Hisashi, N. Mueller, K. Yamada, J. L. Greenstein, R. J. Hawley, C. Patience, M. Awwad, J. A. Fishman, S. C. Robson, H. J. Schuurman, D. H. Sachs, and D. K. Cooper. 2005. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat. Med.* 11:29-31.
208. Yamada, K., K. Yazawa, A. Shimizu, T. Iwanaga, Y. Hisashi, M. Nuhn, P. O'Malley, S. Nobori, P. A. Vagefi, C. Patience, J. Fishman, D. K. Cooper, R. J. Hawley, J. Greenstein, H. J. Schuurman, M. Awwad, M. Sykes, and D. H. Sachs. 2005. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat. Med.* 11:32-34.
209. Baumann, B. C., P. Forte, R. J. Hawley, R. Rieben, M. K. Schneider, and J. D. Seebach. 2004. Lack of galactose-alpha-1,3-galactose expression on porcine endothelial cells prevents complement-induced lysis but not direct xenogeneic NK cytotoxicity. *J. Immunol.* 172:6460-6467.
210. Inverardi, L. and R. Pardi. 1994. Early events in cell-mediated recognition of vascularized xenografts: cooperative interactions between selected lymphocyte subsets and natural antibodies. *Immunol. Rev.* 141:71-93.
211. Rieben, R. and J. D. Seebach. 2005. Xenograft rejection: IgG1, complement and NK cells team up to activate and destroy the endothelium. *Trends Immunol.* 26:2-5.
212. Raulet, D. H., R. E. Vance, and C. W. McMahon. 2001. Regulation of the natural killer cell receptor repertoire. *Annu. Rev. Immunol.* 19:291-330.
213. Garcia, P., M. Llano, A. B. de Heredia, C. B. Willberg, E. Caparros, P. Aparicio, V. M. Braud, and M. Lopez-Botet. 2002. Human T cell receptor-mediated recognition of HLA-E. *Eur. J. Immunol.* 32:936-944.
214. Ulbrecht, M., A. Couturier, S. Martinozzi, M. Pla, R. Srivastava, P. A. Peterson, and E. H. Weiss. 1999. Cell surface expression of HLA-E: interaction with human beta2-microglobulin and allelic differences. *Eur. J. Immunol.* 29:537-547.
215. Miller, J. D., D. A. Weber, C. Ibegbu, J. Pohl, J. D. Altman, and P. E. Jensen. 2003. Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2. *J. Immunol.* 171:1369-1375.
216. Strong, R. K., M. A. Holmes, P. Li, L. Braun, N. Lee, and D. E. Geraghty. 2003. HLA-E allelic variants. Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. *J. Biol. Chem.* 278:5082-5090.
217. Ulbrecht, M., T. Honka, S. Person, J. P. Johnson, and E. H. Weiss. 1992. The HLA-E gene encodes two differentially regulated transcripts and a cell surface protein. *J. Immunol.* 149:2945-2953.
218. Maier, S., M. Grzeschik, E. H. Weiss, and M. Ulbrecht. 2000. Implications of HLA-E allele expression and different HLA-E ligand diversity for the regulation of NK cells. *Hum. Immunol.* 61:1059-1065.
219. Dulat, H. J., W. Baars, K. Wonigeit, and R. Schwinzer. 2001. Target cells expressing CD95L are protected from lysis mediated by alloactivated T cells but are killed by resting NK cells. *Transplant. Proc.* 33:262-263.
220. Pacasova, R., S. Martinozzi, H. J. Boulouis, M. Ulbrecht, J. C. Vieville, F. Sigaux, E. H. Weiss, and M. Pla. 1999. Cell-surface expression and alloantigenic function of a human nonclassical class I molecule (HLA-E) in transgenic mice. *J. Immunol.* 162:5190-5196.
221. Besenfelder, U., J. Modl, M. Muller, and G. Brem. 1997. Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs. *Theriogenology.* 47:1051-1060.
222. Engler-Blum, G., M. Meier, J. Frank, and G. A. Muller. 1993. Reduction of background problems in nonradioactive northern and Southern blot analyses enables higher sensitivity than 32P-based hybridizations. *Anal. Biochem.* 210:235-244.
223. Menier, C., B. Saez, V. Horejsi, S. Martinozzi, I. Krawice-Radanne, S. Bruel, C. Le Danff, M. Reboul, I. Hilgert, M. Rabreau, M. L. Larrad, M. Pla, E. D. Carosella, and N. Rouas-Freiss. 2003. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum. Immunol.* 64:315-326.
224. Maier, S., C. Tertilt, N. Chambron, K. Gerauer, N. Huser, C. D. Heidecke, and K. Pfeffer. 2001. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. *Nat. Med.* 7:557-562.
225. Baldwin, W. M., III, C. P. Larsen, and R. L. Fairchild. 2001. Innate immune responses to transplants: a significant variable with cadaver donors. *Immunity.* 14:369-376.

226. Coudert, J. D., C. Coureau, and J. C. Guery. 2002. Preventing NK cell activation by donor dendritic cells enhances allospecific CD4 T cell priming and promotes Th type 2 responses to transplantation antigens. *J. Immunol.* 169:2979-2987.
227. Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *J. Exp. Med.* 195:327-333.
228. McDouall, R. M., P. Batten, A. McCormack, M. H. Yacoub, and M. L. Rose. 1997. MHC class II expression on human heart microvascular endothelial cells: exquisite sensitivity to interferon-gamma and natural killer cells. *Transplantation* 64:1175-1180.
229. Kummer, J. A., P. C. Wever, A. M. Kamp, I. J. ten Berge, C. E. Hack, and J. J. Weening. 1995. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. *Kidney Int.* 47:70-77.
230. Blancho, G., F. Buzelin, J. Dantal, M. Hourmant, D. Cantarovich, R. Baatard, M. Bonneville, H. Vie, L. Bugeon, and J. P. Soulillou. 1992. Evidence that early acute renal failure may be mediated by CD3- CD16+ cells in a kidney graft recipient with large granular lymphocyte proliferation. *Transplantation* 53:1242-1247.
231. Vampa, M. L., P. J. Norman, L. Burnapp, R. W. Vaughan, S. H. Sacks, and W. Wong. 2003. Natural killer-cell activity after human renal transplantation in relation to killer immunoglobulin-like receptors and human leukocyte antigen mismatch. *Transplantation* 76:1220-1228.
232. Oertel, M., K. Kohlhaw, H. M. Diepolder, S. Schroder, R. Schwarz, A. Tannapfel, J. Mossner, J. Hauss, and F. Berr. 2001. Alloreactivity of natural killer cells in allogeneic liver transplantation. *Transplantation* 72:116-122.
233. Manilay, J. O. and M. Sykes. 1998. Natural killer cells and their role in graft rejection. *Curr. Opin. Immunol.* 10:532-538.
234. Gourlay, W. A., W. H. Chambers, A. P. Monaco, and T. Maki. 1998. Importance of natural killer cells in the rejection of hamster skin xenografts. *Transplantation* 65:727-734.
235. Candinas, D., S. Belliveau, N. Koyamada, T. Miyatake, P. Hechenleitner, W. Mark, F. H. Bach, and W. W. Hancock. 1996. T cell independence of macrophage and natural killer cell infiltration, cytokine production, and endothelial activation during delayed xenograft rejection. *Transplantation* 62:1920-1927.
236. Yim, D., J. Sotiriadis, K. S. Kim, S. C. Shin, H. B. Jie, M. F. Rothschild, and Y. B. Kim. 2002. Molecular cloning, expression pattern and chromosomal mapping of pig CD69. *Immunogenetics.* 54:276-281.
237. Crew, M. D., M. J. Cannon, B. Phanavanh, and C. N. Garcia-Borges. 2005. An HLA-E single chain trimer inhibits human NK cell reactivity towards porcine cells. *Mol. Immunol.* 42:1205-1214.
238. Strong, R. K., M. A. Holmes, P. Li, L. Braun, N. Lee, and D. E. Geraghty. 2003. HLA-E allelic variants. Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. *J. Biol. Chem.* 278:5082-5090.

7.2. List of Abbreviations

aa	amino acid
Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
αGAL	galactose-α-1,3-galactose
α1,3GT	α-1,3-galactosyltransferase
AVR	acute vascular rejection
β2m	β2-microglobulin
CMV	human cytomegalovirus
EC	endothelial cell
E:T ratio	effector-to-target-ratio
GPI	glycosylphosphatidyl-inositol
HAR	hyperacute rejection
HLA	human leukocyte antigen
huβ2m	human β2m
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILT	Ig-like transcript
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer-cell Ig-like receptor
KO	knockout
mAb	monoclonal antibody
MFIR	geometric mean fluorescence intensity ratio
MHC	major histocompatibility complex
MIC	MHC class I chain-related protein
NAb	natural antibody
NCR	natural cytotoxicity receptor
NK	natural killer
PAEC	porcine aortic endothelial cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pEC	porcine endothelial cell
PECAM	platelet/endothelial cell adhesion molecule
PERV	porcine endogenous retrovirus
pMIC2	porcine MIC2
PMN	polymorphonuclear neutrophil
pULBP1	porcine ULBP1
SCT	single chain trimer
shRNA	short hairpin RNA
siRNA	short interfering RNA
SLA	swine leukocyte antigen
TNF	tumor necrosis factor
ULBP	UL16-binding protein
VCAM	vascular cell adhesion molecule

7.3. Curriculum Vitae

Name:	Benjamin Geoffrey LILIENFELD, MSc.
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Education

02/2004-02/2007	**Ph.D.-student** at the Faculty of Science, University of Zurich, PhD thesis in the Laboratory for Transplantation Immunology at the University Hospital in Zurich (PD Dr. J. Seebach).
10/1999-01/2004	**Biology study** at the University of Zurich. Major subject: molecular biology, minor subjects: biochemical immunology, physical chemistry Diploma thesis: "Characterization of CD46 as a putative receptor for species B adenoviruses".
08/1992-01/1999	High school in Urdorf (Zurich, Switzerland)

Continuing education

05/2005	Course in "Effective Presentations" at the speech center of the University of Zurich.
1/2005	Course in "Writing Research Papers for Biomedical Publications" at the speech center of the University of Zurich.

Awards

01/2007	3rd price of the Swiss Transplantation Society for the publication entitled "Porcine UL-16 binding protein 1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D.", Journal of Immunology, 2006. (Swiss Transplant Research Award)
07/2006	Travel Grant of the Hartmann Müller Foundation for the 4th International Conference on Innate Immunity, Corfu, Greece (June 4-9, 2006)

01/2006	3rd price of the Swiss Transplantation Society for the publication entitled “Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D”, Journal of Immunology, 2005. (Swiss Transplant Research Award)
09/2005	Travel Grant of the Transplantation Society for the 8th International Xenotransplantation Congress and 2nd International Symposium on ABO Incompatibility in Transplantation, Göteborg, Sweden (September 10-14, 2005)
08/2005-07/2006	Grant of the Roche Research Foundation for 12 months of the PhD study
08/2004-07/2005	Grant of the Hartmann Müller Foundation for 12 months of the PhD study

Publication list

1. Lilienfeld B*, Sirena D*, Eisenhut M*, Kalin S, Boucke K, Beerli RR, Vogt L, Ruedl C, Bachmann MF, Greber UF, Hemmi S. The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3. **Journal of Virology** 2004, 78:4454-62. * B.L., D.S., and M.E. contributed equally

2. Lilienfeld BG und Seebach JD. Of Mice and Men – Mäuse mit humanem Immunsystem. **Forum Med Suisse** 2004, 44:1122

3. Baumann BC, Schneider MKJ, Lilienfeld BG, Antsiferova MA., Rhyner DM, Hawley RJ, Seebach JD. Endothelial cells derived from pigs lacking Galα(1,3)Gal: no reduction of human leukocyte adhesion and NK cytotoxicity. **Transplantation** 2005, 79: 1067-1072

4. Lilienfeld BG*, Forte P*, Baumann BC, and Seebach JD. Human NK cytotoxicity against porcine cells is triggered by NKp44 and NKG2D. **Journal of Immunology** 2005, 175: 5463-5470. *Both authors contributed equally

5. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine UL-16 binding protein 1 (pULBP1) expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. **Journal of Immunology** 2006, 177: 2146-2152

6. Lilienfeld BG, Forte P, Baumann BC, Crew MD, and Seebach JD. Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human NK-mediated cytotoxicity. **Xenotransplantation** 2007, 14: 126-134

7. Weiss EH, Lilienfeld BG, Müller S, Herbach N, Keßler B, Wanke R, Schwinzer R, Seebach JD, Wolf E and Brem G. Cells from HLA-E/β2-microglobulin expressing pigs are effectively protected against human natural killer cell cytotoxicity. *manuscript submitted*

8. Lilienfeld BG, Schildknecht A, Imbach L, Schneider MKJ, Mueller NJ, and Seebach JD. Characterization of porcine UL-16 binding protein 1 (pULBP1) endothelial cell surface expression using a novel anti-pULBP1 monoclonal antibody. *manuscript in preparation*

Congresses

1. Sirena D, Lilienfeld B, Beerli R, Bachmann M, Eisenhut M, Greber UF, Hemmi S. Molecular characterization of a receptor for human adenovirus B serotypes. European Life scientist organization congress, September 21-24, 2003, Dresden, Germany. (abstract, poster presentation)
2. Forte P, Lilienfeld B, and Seebach JD. Role of activation receptors in the cytotoxic activity of human NK cell against porcine cells. 8th Annual Meeting of the Society for Natural Immunity and 20th International Natural Killer Cell Workshop, April 24–28, 2004, Noordwijkerhout, The Netherlands. (abstract, poster presentation)
3. Forte P, Baumann BC, Lilienfeld BG, Schneider MKJ, Seebach JD. Prevention of NK cell-mediated cytotoxicity in pig-to-human xenotransplantation. 20th International congress of the transplantation society, September 5-10, 2004, Vienna, Austria. (abstract, oral presentation) *Transplantation* 2004, 78 (Suppl.2): S21 (abstract)
4. Lilienfeld BG, Forte P, Baumann BC, Seebach JD. Human NK cytotoxicity against porcine endothelial cells is triggered by NKp44 and NKG2D. Annual Congress of the SSAI/SSPT, March 3-4, 2005, Berne, Switzerland. (abstract, oral presentation) *Swiss Med Wkly* 2005, 135 (Suppl.144): S3 (abstract)
5. Lilienfeld BG, Forte P, Baumann BC, Seebach JD. Human NK cytotoxicity against porcine endothelial cells is triggered by NKp44 and NKG2D. 4th Day of Clinical Research, March 10-11, 2005, Zurich, Switzerland. (abstract, oral presentation)
6. Forte P, Baumann BC, Lilienfeld BG, Schneider MKJ, Seebach JD. Prevention of NK cell-mediated cytotoxicity in pig-to-human xenotransplantation. 3. Treffen des Arbeitskreises Transplantationsimmunologie der Deutschen Gesellschaft für Immunologie (DGFI), March 11-12, 2005, Würzburg, Germany. (abstract, oral presentation)
7. Lilienfeld BG, Forte P, Baumann BC, Seebach JD. Human NK cytotoxicity against porcine endothelial cells is triggered by NKp44 and NKG2D. 17th Meeting of the Swiss Immunology Ph.D. Students, March 30 - April 1, 2005 at Schloss Wolfsberg, Switzerland. (abstract, poster presentation)
8. Lilienfeld BG, Forte P, Baumann BC, Seebach JD. Human NK cytotoxicity against porcine endothelial cells is triggered by NKp44 and NKG2D. 8th International Xenotransplantation Congress and 2nd International Symposium on ABO Incompatibility in Transplantation, September 10-14, 2005, Göteborg, Sweden. (abstract, oral presentation) *Xenotransplantation* 2005, 12 (5): O10:5 (abstract)

9. Schneider MKJ, Antisferova MA, Rhyner DM, Lilienfeld BG, Baumann BC, Seebach JD. Recruitement of human leukocytes to porcine endothelial cells expressing or lacking Gal-alpha (1,3) Gal. 8th International Xenotransplantation Congress and 2nd International Symposium on ABO Incompatibility in Transplantation, September 10-14, 2005, Göteborg, Sweden. (abstract, oral presentation) *Xenotransplantation* 2005, 12 (5): O10:2 (abstract)
10. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine ULBP1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. 18th Meeting of the Swiss Immunology Ph.D. Students, March 20-22, 2006 at Schloss Wolfsberg, Switzerland. (abstract, oral presentation)
11. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine ULBP1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. 5th Day of Clinical Research, March 23-24, 2006, Zurich, Switzerland. (abstract, oral presentation)
12. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine ULBP1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. Annual Congress of the SSAI/SSPT, March 30-31, 2006, Zurich, Switzerland. (abstract, poster presentation) *Swiss Med Wkly* 2006, 136 (Suppl. 149), S32 (abstract)
13. Lilienfeld BG, Forte P, Baumann BC, Crew MD, and Seebach JD. Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human NK-mediated cytotoxicity. Annual Congress of the SSAI/SSPT, March 30-31, 2006, Zurich, Switzerland. (abstract, poster presentation) *Swiss Med Wkly* 2006, 136 (Suppl. 149), S32 (abstract)
14. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine ULBP1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. 4th International Conference on Innate Immunity, June 4-9, 2006, Corfu, Greece. (abstract, poster presentation)
15. Forte P, Lilienfeld BG, Baumann BC, Crew MD, Schneider MKJ, and Seebach JD. HLA Expression on Porcine Endothelial Cells to Prevent Human Anti-Porcine NK Cell Reactions. 9. Minisymposium Xenotransplantation, DFG-Transregio Research Group Xenotransplantation (FOR 535), June 8-9, 2006, Robert Koch Institut, Berlin, Germany. (abstract, oral presentation)
16. Lilienfeld BG, Garcia-Borges C, Crew MD, and Seebach JD. Porcine ULBP1 expressed on the surface of endothelial cells activates human NK cytotoxicity via NKG2D. World Transplant Congress, July 23-27, 2006, Boston, USA. (abstract, poster presentation)
17. Lilienfeld BG, Forte P, Baumann BC, Crew MD, and Seebach JD. Transgenic expression of HLA-E single chain trimer protects porcine endothelial cells against human NK-mediated cytotoxicity. World Transplant Congress, July 23-27, 2006, Boston, USA. (abstract, poster presentation)

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